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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Cancer is thought to be a result of both uncontrolled cell proliferation and the failure of cells to undergo apoptosis. Akt is a serine/threonine protein kinase that has been shown to mediate cell survival. Akt is activated by a number of growth factors involved in mammary gland development. Our initial studies suggest that the hormone prolactin, which is involved in mammary gland development, can induce activation of Akt. Studies in this update demonstrate that prolactin may induce Akt activation in the prolactin-dependent cell line, Nb2, via src-like kinases, the adapter protein Cbl, and PI3K. The primary goal of this grant is to examine the potential roles of Akt in the mammary gland and in inducing mammary cancer. Examination of transgenic mice that express a constitutively active form of Akt reveals that Akt may suppress apoptosis in mammary epithelial cells and, in addition, may be involved in milk lipid synthesis during lactation. In addition, the expression of this transgene in the mammary gland may induce a preneoplastic state in the mammary gland in which the mammary gland may be more susceptible to tumor development.

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Introduction

Although cancer is typically thought of as the result of uncontrolled cellular proliferation, it can also involve the inability of cells to undergo apoptosis at the correct time. Therefore, numerous studies have focused on the roles of various anti-apoptotic molecules in the induction of cancer. Akt is a serine/threonine protein kinase that has been shown to suppress apoptosis in response to a wide variety of stimuli (1-7). In addition, overexpression of Akt has been identified in certain types of cancers, such as gastric adenocarcinomas (8), and ovarian and pancreatic carcinomas (9-12). Our initial studies focused on the ability of prolactin to induce activation of Akt and on the identification of signaling pathways that mediate this activation. Results described in this summary demonstrate that prolactin may induce activation of Akt through a src-like kinase→Cbl→ PI3K→ Akt pathway. Because Akt is activated in response to a number of growth factors that are involved in mammary gland development (6;13-18), we hypothesized that overexpression of Akt in the mammary gland may result in the suppression of apoptosis, possibly leading to tumorigenesis. We have generated mice that express a constitutively active from of Akt in the mammary gland under the control of the mouse mammary tumor virus (MMTV) promoter. Upon examination of the effects of this transgene on the mammary gland during development, we have shown that Akt can suppress apoptosis during mammary gland involution. Surprisingly, our data also suggest that Akt may also be involved in lipid synthesis during pregnancy and lactation. Finally, although the presence of myr-Akt in the mammary gland does not result in high levels of spontaneous tumors, it is possible that Akt overexpression may result in the induction of a preneoplastic state and in the presence of other mutations, may result in tumorigenesis.

Body

The goal of Aim 1 is to identify the upstream signaling molecules that mediate prolactin-induced activation of Akt. Because tyrosine phosphorylation of the prolactin receptor is thought to be critical for the activation of signaling pathways by prolactin (19;20), we obtained a panel of receptor mutants in which the tyrosine residues were sequentially mutated to phenylalanine residues (21). Each receptor contains eight tyrosine to phenylalanine mutations, with only one remaining tyrosine residue. The mutant receptors were stably transfected into 32Dcl3 cells, selected with 1 mg/ml neomycin, and single cell derived clones were isolated by growth in soft agar. Each line was examined for the ability to activate janus kinase 2 (JAK2) following stimulation of the cells with prolactin. Cell lines containing each mutant were identified as determined by the ability of prolactin stimulation to induce phosphorylation of JAK2 (data not shown). Each cell line was then examined for the ability to activate Akt following prolactin stimulation. The only cell lines that were capable of activating Akt were the lines containing the wild-type receptor (32D/long) and the mutant receptor containing the tyrosine 580 residue (32D/8FY580) (Figure 1), suggesting that this tyrosine residue may be responsible for mediating Akt activation. There are two bands present in the immunoblot of the 32D/8FY580 cells. The higher molecular weight band represents phosphorylated Akt and the lower molecular weight band is likely to be a non-specific band because it is present in both lanes. The activation of Akt observed in the 32D/8FY580 cells is not as robust as the activation in cells containing the wild-type receptor. One possibility for this observation is that there is more than one tyrosine residue required for Akt activation. Another possibility is that the number of receptors expressed on the cell surface is not high enough to mediate strong activation of Akt. These constructs are being expressed from a cytomegalovirus (CMV) promoter, which is not a highly active promoter in hematopoietic cells. Therefore, these constructs are in the process of being subcloned into another vector that contains an RSV promoter. In addition, a hemagluttunin (HA) epitope tag will be added to the carboxy-terminal end of the receptor mutants so that we can compare expression levels of the receptors between the different cell lines.
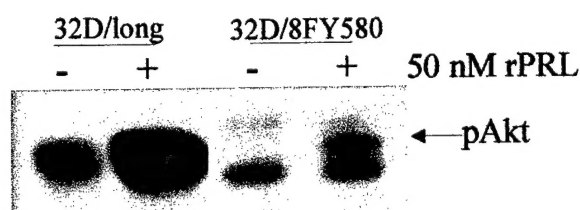

Figure 1: Activation of Akt by 32D cells expressing the wild-type and 8FY580 prolactin receptors. 32D/long and 32D/8FY580 cells were cultured in media containing 2% charcoal stripped serum for 18 hours and were then stimulated with 50 nM rPRL for 30 minutes. The cells were lysed, and equal amounts of proteins were analyzed on an 8% polyacrylamide gel. Immunoblot analysis was performed with an anti-phospho-Akt antibody.

As described in last year's annual summary, we have shown that prolactin may mediate activation of Akt through src-like kinases and phosphatidylinositol 3-kinase (PI3K) in Nb2 cells using specific inhibitors. As described in the grant proposal, studies from our lab have demonstrated that prolactin may mediate activation of PI3K through binding of the p85 subunit of PI3K to a critical phosphotyrosine on the adapter protein Cbl (22). In the previous update, we demonstrated that treatment of the Nb2 cell line with a specific inhibitor of src-like kinases, PP1, results in decreased Akt phosphorylation. We have also examined the phosphorylation state of Cbl following PP1 treatment of Nb2 cells. As shown in Figure 2, treatment of cells with PP1 results in decreased tyrosine phosphorylation of Cbl following prolactin stimulation. We believe that these studies as well as the studies described in the previous update suggest that prolactin may mediate activation of Akt through a src-like kinase→Cbl→PI3K→Akt pathway in Nb2 cells. However, further experiments are required to confirm these results. Experiments utilizing

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mutant constructs of the various components of this pathway were described in the original proposal. However, we have not yet developed the optimal method for expressing these constructs in Nb2 cells. The transfection efficiency of Nb2 cells following electroporation has proven to be low. As described in the next section, we have also attempted to express constructs in Nb2 cells using the adenoviral transduction method, which has also not produced high levels of expression. We are currently attempting to express constructs in the Nb2 cells using retroviral transduction.
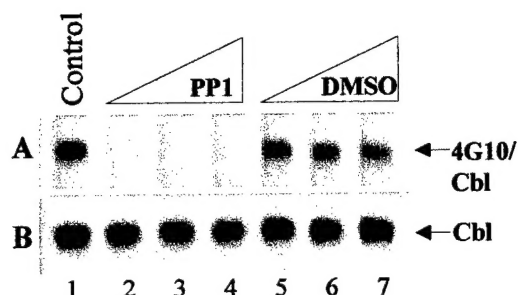

Figure 2: Inhibition of Cbl phosphorylation following PP1 treatment of Nb2 cells. Nb2 cells were incubated overnight in the absence of PRL. Nb2 cells were then stimulated with PRL alone (lane 1), and incubated in the presence of either PP1 (25, 50, 100 µM) (lanes 2-4) or equivalent amounts of DMSO (lanes 5-7) for 4 hours. Cbl was immunoprecipitated using an anti-Cbl antibody and analyzed on a 7.5% SDS-PAGE gel. A) The proteins were transferred to a membrane and immunoblotted with phosphotyrosine antisera (4G10). B) The immunoblot in A was re-probed with an antibody to Cbl to indicate equal loading.

The goal of Aim 2 is to examine the effects of a constitutively active form of Akt on cell survival in various cell lines. To examine this, we had proposed to stably express constitutively active Akt (myr-Akt) in Nb2 cells and 32Dcl3 cells expressing the human form of the prolactin receptor and examine the effects of this construct on cell survival following prolactin withdrawal. Despite extensive efforts to produce a cell line that stably expresses constitutively active Akt, we have been unable to generate this line. However, we were able to generate stable cell lines that express wild-type Akt (data not shown). We have also attempted to express myr-Akt in Nb2 cells using adenoviral transduction. Because Nb2 cells do not readily transduce with adenovirus, we generated stable cell lines that express the coxsackie and adenovirus receptor (CAR) (16) and have shown that transduction of these cells with green fluorescent protein (GFP) results in expression in more than 90% of the cells (data not shown). However, attempts to express high levels of myr-Akt in these cells have resulted in toxicity. In contrast, studies shown in the previous update demonstrated that adenoviral transduction of myr-Akt into the mammary epithelial cell line, HC-11, results in multiplicity of infection (MOI)-dependent expression of the myr-Akt construct. In addition, we have been able to transfect these cells with myr-Akt cDNA, which also results in detectable levels of expression (data not shown). Therefore, experiments are in progress to determine the effects of myr-Akt expression on cell survival in mammary epithelial cell lines following either growth factor withdrawal or detachment from basement membrane.

The goal of Aim 3 is to determine the effects of myr-Akt expression in mammary glands of transgenic mice. In the previous update, generation of the MMTV-myr-Akt transgenic mice, expression of myr-Akt in the mammary gland, and the effects of myr-Akt on the involution process were discussed. These studies have been continued by examining mammary gland morphology in virgin mice, and during pregnancy and lactation using either whole mount or histological analyses. Data presented in the previous update suggested that expression of myr-Akt is not detectable until lactation in mammary glands from transgenic mice. However, further analysis demonstrates that following longer exposure times of the immunoblots, we can detect transgene expression in mammary glands from virgin and pregnant animals (Figure 3). As observed in Figure 3, we consistently detect two bands in these immunoblots. Further analysis has demonstrated that re-probing these blots with an anti-Akt antibody recognizes both bands;

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however, re-probing these blots with an anti-phospho-Akt antibody only recognizes the lower molecular weight band (data not shown). Therefore, it is possible that the presence of the two bands may represent a shift due to phosphorylation of the transgene. As observed in Figure 3A, myr-Akt expression in mammary glands from virgin mice appears to be variable. This variation is likely due to the fluctuating levels of hormones during the estrous cycle, since the MMTV promoter is hormonally responsive. During pregnancy, it appears that expression levels of myr-Akt increase as pregnancy progresses (Figure 3B), and the peak of myr-Akt expression occurs during lactation, as demonstrated in the previous update.
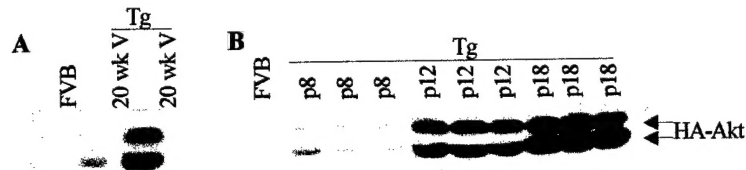

Figure 3: Expression of myr-Akt protein in mammary glands from virgin and pregnant transgenic mice. Protein extracts were prepared from mammary glands of wild-type (FVB) and transgenic mice (Tg) and immunoblotted with an anti-HA antibody. A) Myr-Akt expression in mammary glands from wild-type (FVB) and transgenic (Tg) mice at 20 weeks of age. B) Myr-Akt expression in mammary glands from wild-type (FVB) and transgenic (Tg) mice at days 8, 12, and 18 of pregnancy.

Analysis of mammary gland morphology of virgin mice using both whole mounts and histology demonstrates that there are no apparent differences between mammary glands of wild-type mice and mammary glands of transgenic mice at this stage (data not shown). Mammary glands from wild-type and transgenic mice were also analyzed at days 8, 12, and 18 of pregnancy. Lower magnification views of the mammary glands are used to examine overall alveolar development (Figure 4 A, C, E, and G) and higher magnification views are used to examine individual alveoli for differences (Figure 4 B, D, F, and H). At day 8 of pregnancy, alveolar development appears comparable between mammary glands from wild-type mice and mammary glands from transgenic mice (data not shown). Alveolar development also appears comparable at day 12 of pregnancy between mammary glands from wild-type (Figure 4A) and transgenic mice (Figure 4C). However, examination of sections at higher magnification reveals some differences in the individual alveoli in mammary glands from transgenic mice compared to those from wild-type mice. In the alveoli from wild-type mice, small lipid droplets are apparent in the alveoli during mid-pregnancy (arrow, Figure 4B). The lipid droplets are also apparent in
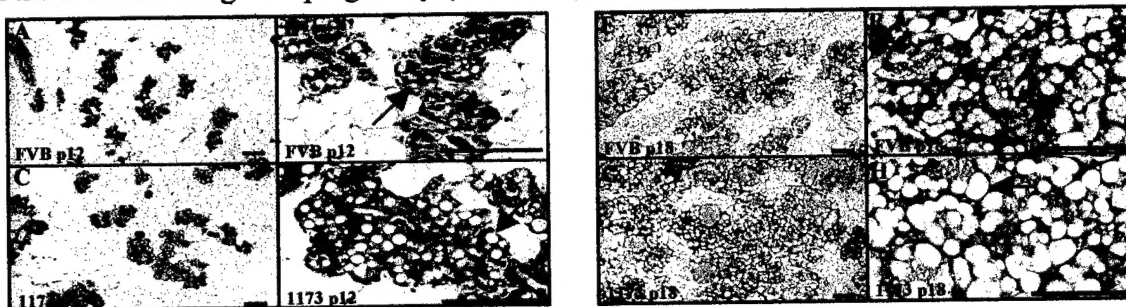
Figure 4: Mammary gland development during pregnancy in MMTV-myr-Akt transgenic mice. Mammary glands were removed from both wild-type (FVB) and transgenic (1173) mice at days 12 and 18 of pregnancy (p12 and p18), embedded in paraffin, and sections were stained with hematoxylin and eosin. A, B) Mammary glands sections from FVB mice at day 12 of pregnancy. C, D) Mammary gland sections from transgenic mice at day 12 of pregnancy. E, F) Mammary gland sections from FVB mice at day 18 of pregnancy. G, H) Mammary gland sections from transgenic mice at day 18 of pregnancy. Magnification bars represent 100 microns. Milk lipid droplets are indicated by the arrows in panels B, D, F, and H.

the alveoli from transgenic mice (arrow, Figure 4D); however, they appear to be larger in size than those observed in the alveoli from wild-type mice. The increased size of lipid droplets is also observed at day 18 of pregnancy in alveoli in transgenic mice (arrow, Figure 4H) compared to alveoli in wild-type mice (arrow, Figure 4F). Overall development of the alveoli does not seem to be affected in the transgenic mice (Figure 4G) compared to the wild-type mice (Figure 4E). These data suggest that in addition to suppressing apoptosis, myr-Akt may be involved in regulating the synthesis and/or secretion of lipid droplets in developing alveoli during pregnancy.

To further characterize the MMTV-myr-Akt transgenic mice, mammary gland morphology during lactation was examined using hematoxylin and eosin stained sections from both wild-type and transgenic mice. At day 9 of lactation in mammary glands from transgenic mice, the alveoli have filled the mammary fat pad (Figure 5C), but they appear larger and distended compared to the alveoli from wild-type mice (Figure 5A). In addition, the lipid droplets observed in the alveoli are larger in size and can be found around the edges of the lumen, possibly in the process of being secreted from the epithelial cells (arrows, Figure 5B,D), as well as in the lumen (arrowhead, Figure 5D). In addition, there appears to be more proteinaceous material in the alveolar lumina (asterisk, Figure 5D) compared to wild-type alveoli (asterisk, Figure 5B), suggesting that milk stasis may be occurring. Similarly, at day 15 of lactation, the alveoli from transgenic mice remain distended (Figure 5G) compared to the alveoli from wild-type mice (Figure 5E). The lipid droplets are also larger and the proteinaceous material persists in alveoli from transgenic mice (Figure 5H) compared to wild-type mice (Figure 5F).

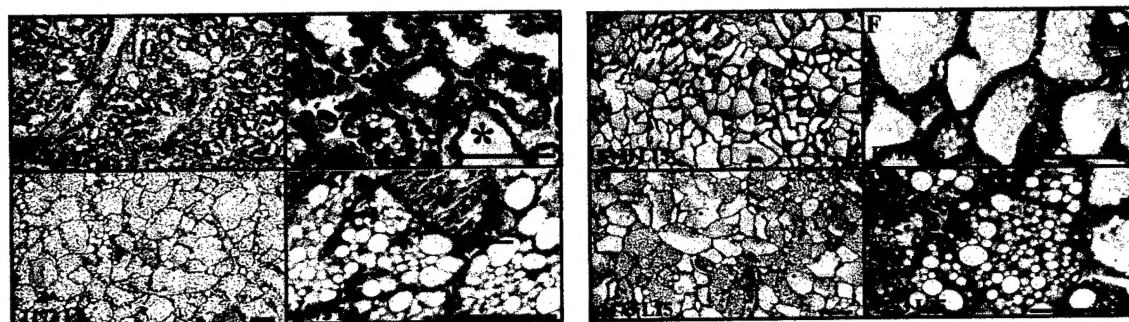**Figure 5: Increased size of lipid droplets in mammary glands from MMTV-myr-Akt transgenic mice during lactation.** Mammary glands were removed from both wild-type (FVB) and transgenic (1173) mice at days 9 and 15 of lactation (L9 and L15), embedded in paraffin, and sections were stained with hematoxylin and eosin. A,B) Mammary gland sections from FVB mice at day 9 of lactation. Milk lipid droplets (arrow) are observed in the lumen (asterisk) in panel B. C,D) Mammary gland sections from transgenic mice at day 9 of lactation. Milk lipid droplets (arrow) are observed in the lumen (asterisk) in panel D. E,F) Mammary glands from FVB mice at day 15 of lactation. G,H) Mammary glands from transgenic mice at day 15 of lactation. Magnification bars represent 100 microns.

We further examined the lactation phenotype by analyzing the composition of milk from the transgenic mice. Milk was collected from both wild-type and transgenic mice and analyzed for protein and fat composition. Although protein composition of milk from transgenic mice is comparable to that from wild-type mice (data not shown), there is a significant increase in the percentage of fat in milk from transgenic mice (67%) compared to milk from wild-type mice (25%) (Figure 6). The lactation defect observed in transgenic mice, which was described in the previous update, is likely due to the high percentage of milk fat, which makes the milk difficult for newborn pups to extract from the mammary gland. These results suggest that expression of

myr-Akt in mammary epithelial cells is affecting lipid synthesis and/or secretion, consistent with the observations made during pregnancy.
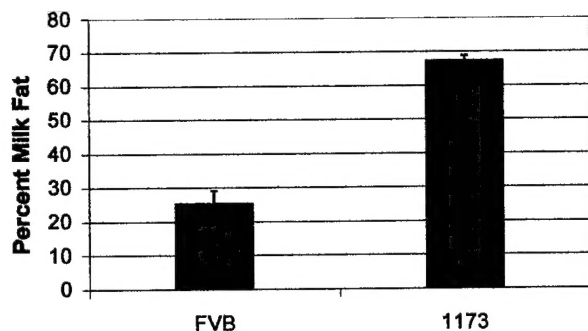

Figure 6: Increased fat content and normal protein composition in milk from MMTV-myr-Akt transgenic mice. Milk was collected from wild-type (FVB) and transgenic (1173) mice at day 10 of lactation. The percentage of milk fat was calculated following centrifugation of whole milk. Error bars represent S.E.M.

Data described in the previous update demonstrate that expression of myr-Akt results in delayed involution and a delay in the onset of apoptosis following forced weaning. Further experiments were performed to characterize this phenotype, which are described in detail in the attached manuscript (Schwertfeger et al., 2001). We have observed that although the mammary glands eventually regress, there appears to be more epithelium present in mammary glands from transgenic mice compared to those from wild-type mice (see Schwertfeger et al., 2001, Figure 4). To further examine this observation, the amount of epithelium was quantitated at day 21 of involution. There is approximately a 2-fold increase in the amount of epithelium present in mammary glands from transgenic mice compared to those from wild-type mice. It is possible that increased epithelium may result in the subsequent development of hyperplasias in mammary glands from transgenic mice following the first or subsequent pregnancies, however, this possibility remains to be examined.

The formation of spontaneous tumors in MMTV-myr-Akt transgenic mice appears to be infrequent. Two poorly differentiated mammary adenocarcinomas have been identified during pregnancy (data not shown) and during involution (Figure 7A) that express high levels of myr-Akt transgene (Figure 7B). In addition, two hyperplasias have been identified in mammary glands during pregnancy and involution (data not shown), although the presence of myr-Akt transgene in these lesions has not yet been examined. Although it is apparent that expression of myr-Akt is not strong enough to induce tumorigenesis on its own, it is possible that expression of myr-Akt may induce a preneoplastic state in the mammary gland and that other events are required to induce tumorigenesis. As mentioned above, development of hyperplasias and tumors following one or multiple pregnancies remains to be examined, which should provide more insights into the potential roles of Akt in these processes in the mammary gland.
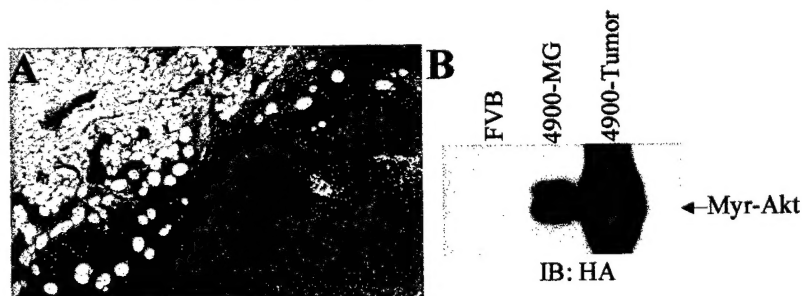

Figure 7: Adenocarcinoma from the mammary gland of a transgenic mice. A) Hematoxylin/eosin stained section of a mammary gland tumor (*) from a transgenic mouse. B) Expression of the myr-Akt transgene in mammary glands from wild-type FVB) and transgenic (4900-MG) mice and in the tumor (4900-Tumor). Protein extracts were immunoblotted with the anti-HA antibody.

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Key Research Accomplishments

- Prolactin-induced Akt activation may be mediated by the tyrosine 580 residue on the prolactin receptor.
- Experiments using the chemical inhibitor PP1 indicate that prolactin-induced activation of Akt may be mediated through a src-like kinase→Cbl→ PI3K→ Akt pathway.
- Expression of myr-Akt in the transgenic mice is observed in mammary glands from virgin and pregnant mice.
- Analysis of mammary glands from transgenic mice during pregnancy and lactation suggests that Akt may be involved in the lipid synthesis pathway in mammary epithelial cells. In addition, mammary gland involution and the onset of apoptosis is delayed in transgenic mice, suggesting that Akt may suppress apoptosis in the mammary gland.
- More epithelium is present in mammary glands from transgenic mice following weaning, suggesting that not all of the epithelial cells are undergoing apoptosis during involution.
- A small number of hyperplasias and tumors have been identified in mammary glands from transgenic mice, suggesting that Akt may induce a preneoplastic state in the mammary gland, although its expression alone is probably not sufficient for the induction of tumorigenesis.

Reportable Outcomes

Abstracts:
Schwertfeger, K.L., Richert, M.M., McManaman, J.L., Lewis, M.T., and Anderson, S.M. "Delayed Mammary Gland Involution and a Defect in Lactation in Transgenic Mice Expressing Activated Akt.".
Presented as a poster at the Mammary Gland Gordon Conference, June, 2001.

Schwertfeger, K.L., McManaman, J.L., Richert, M.M. and Anderson, S.M. "Expression of Constitutively Active Akt in the Mouse Mammary Gland: Effects on Apoptosis and Lipid Synthesis".
Presented as an oral presentation (#53-3) by KLS at the 83rd Meeting of the Endocrine Society, June 2001.

Publications:
Richert, M.M.[*], **Schwertfeger, K.L.**[*], Ryder, J.W., and Anderson, S.M. An Atlas of Mouse Mammary Gland Development. *Journal of Mammary Gland Biology and Neoplasia.* 5(2), 227-241, 2000.
[*]These authors contributed equally

Schwertfeger, K.L., Richert, M.M., and Anderson, S.M. Mammary Gland Involution is Delayed by Activated Akt in Transgenic Mice. *Molecular Endocrinology.* 15(6), 867-881, 2001.

Conclusions

The initial aims outlined in this grant proposal focuses on determining the signaling pathways involved in mediating prolactin-induced activation of Akt and examining the effects of activated Akt on various cell lines. We have preliminary evidence that prolactin can potentially mediate Akt through src-like kinases, Cbl, and PI3K in Nb2 cells. However, these results are based on experiments using chemical inhibitors, which raises questions concerning specificity of the inhibitors. Further experiments will be performed using dominant negative and constitutively active mutants of the various signaling molecules in the proposed pathway to determine whether these molecules are responsible for mediating prolactin-induced activation of Akt in Nb2 cells. In addition, these experiments should also be performed in other cell types to be certain that the molecules involved in this signaling pathway are not cell type specific. These results of these experiments contribute to the scientific understanding of how prolactin and potentially other growth factors induce activation of specific signaling pathways and mediate the suppression of apoptosis.

The final aim of the grant focuses on the generation and characterization of the MMTV-myr-Akt transgenic mice. We have identified mice that express the myr-Akt transgene in the mammary gland throughout development. Our initial experiments, which were described in the previous report, demonstrate that expression of myr-Akt in the mammary gland delays involution and suppresses apoptosis of the mammary epithelial cells, indicating that Akt may suppress apoptosis in the mammary gland. In addition, we have also identified another mammary gland phenoype in the MMTV-myr-Akt mice, which is characterized by an increase in the size of lipid droplets during pregnancy and lactation and increased fat in milk from transgenic mice. Interestingly, Akt has been previously shown to mediate lipid synthesis in 3T3-L1 adipocytes (23-25). Therefore, it appears in addition to the suppression of apoptosis, Akt may also be involved in milk fat synthesis during lactation. Further experiments will be performed to examine the ability of myr-Akt to suppress apoptosis and induce lipid synthesis in mammary epithelial cells in culture. Although the MMTV-myr-Akt transgenic mice do not exhibit high rates of mammary tumors, we have identified a small number of tumors and hyperplasias at different times during development. Therefore, it is unlikely that Akt can induce tumorigenesis on its own, but may induce a preneoplastic state in the mammary gland and may induce tumorigenesis in the presence of other mutations. To further examine the potential roles of Akt in inducing tumorigenesis, we will examine mammary glands following one or multiple pregnancies.

The results from the MMTV-myr-Akt transgenic mice contribute to the scientific understanding of the roles of Akt in the mammary gland. Although we had predicted that expression of the transgene in the mammary gland would likely lead to the suppression of apoptosis, we did not predict the potential role for Akt in lactation. Further analyses of tumor development in these mice will lead to better understanding of the roles of cell survival and possibly oncogene cooperativity in mammary tumor development.

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Delayed Mammary Gland Involution and a Defect in Lactation in Transgenic Mice Expressing Activated Akt. Schwertfeger, K.L., Richert, M.M., McManaman, J.L., Lewis, M.T., and Anderson, S.M.

Apoptosis is known to occur in the mammary gland at several developmental time points including in the terminal end buds during puberty and, most dramatically, during involution following weaning. It is not clear what signaling molecules regulate apoptosis during involution. We have focused upon the anti-apoptotic serine/threonine protein kinase Akt (also known as protein kinase B) which is activated by insulin-like growth factor I, epidermal growth factor, prolactin, and estrogen, all of which are important in regulating mammary gland development. Using immunoblot analysis, we show that Akt is expressed in the virgin, pregnant, lactating, and involuting mammary gland. Immunofluorescence analysis using Akt1 and Akt2 specific antibodies indicate differential expression patterns of these two proteins. To examine the role of Akt in regulating mammary gland involution we have generated transgenic mice which express a constitutively activated mutant of Akt referred to as Myr-Akt under the control of the mouse mammary tumor virus promoter. Founder mice were established and the expression of the transgene has been determined using both RT-PCR and immunoblot analysis. Morphological analysis indicates that involution is delayed in transgenic mice following forced weaning. Secretory alveolar structures remain intact for at least 6 days after withdrawal of the pups, and there is a delay in the onset of epithelial cell apoptosis, demonstrating an effect of activated Akt on tissue remodeling. A less dramatic phenotype was also observed in a second transgenic line corresponding to a reduced level of transgene expression. In addition to suppressed involution, there is an effect on lactation since the growth of pups nursed by transgenic females (as determined by weight) is decreased by nearly 50% compared to litters nursed by normal females over the first eight days of life. Histological analysis and immunofluorescence indicate that lipid droplets in the mammary glands from lactating transgenic mice are much larger than in mammary glands from normal mice, and the composition of milk fat is higher in milk from the transgenic mice. These data suggest that Akt is a critical regulator of mammary involution and may also influence lactation by affecting lipid synthesis and/or secretion.

Expression of Constitutively Active Akt in the Mouse Mammary Gland: Effects on Apoptosis and Lipid Synthesis. Schwertfeger, K.L., McManaman, J.L., Richert, M.M. and Anderson, S.M.

The serine/threonine protein kinase Akt has been shown to suppress apoptosis in a number of systems as well as mediate insulin-stimulated glucose uptake and lipid synthesis. In the mouse mammary gland, Akt is expressed at high levels during lactation, when milk lipid synthesis is high, and decreases at the onset of involution, when apoptosis occurs. This expression pattern suggests potential roles for Akt in both lipid synthesis and suppression of apoptosis in the mammary gland. To examine the role of Akt in mammary gland development, transgenic mice were generated that express a constitutively active form of Akt (myr-Akt) under the control of the MMTV promoter. Immunoblot analysis shows transgene expression throughout mammary gland development, with highest levels of expression during lactation and early in involution. We have shown that expression of the myr-Akt transgene results in delayed involution, consistent with the anti-apoptotic function of Akt. In addition, the transgenic mice exhibit a defect in lactation, indicated by decreased pup weight in litters nursed by transgenic mothers. Histological analysis of mammary glands from pregnant and lactating mice indicates an increased size of milk fat droplets in mammary glands from transgenic mice compared to those from wild-type mice. Although milk protein composition appears normal, the percentage of milk fat is dramatically increased in milk from transgenic mice (67%) compared to milk from wild-type mice (25%). Experiments are currently in progress to examine the downstream effects of Akt that could result in the lactation phenotype. In addition, gene array analysis is being utilized to identify differentially regulated genes during lactation in mammary glands from transgenic and wild-type mice, which should elucidate the downstream effects of Akt in this phenotype. In conclusion, analysis of the expression of myr-Akt in the mammary glands of transgenic mice suggests a dual function for Akt in the mammary gland as both a regulator of apoptosis and a mediator of lipid synthesis.

An Atlas of Mouse Mammary Gland Development

Monica M. Richert,[1,3,4] **Kathryn L. Schwertfeger,**[2,3] **John W. Ryder,**[1] **and Steven M. Anderson**[1,2]

The mouse mammary gland is a complex tissue, which is continually undergoing changes in structure and function. Embryonically, the gland begins with invasion of the underlying fat pad by a rudimentary ductal structure. Postnatal growth occurs in two phases: ductal growth and early alveolar development during estrous cycles, and cycles of proliferation, differentiation, and death that occur with each pregnancy, lactation, and involution. The variety of epithelial structures and stromal changes throughout the life of a mammary gland makes it a challenge to study. The purpose of this histological review is to give a brief representation of the morphological changes that occur throughout the cycle of mouse mammary gland development so that developmental changes observed in mouse models of mammary development can be appreciated.

KEY WORDS: Mammary gland; mouse; duct; alveoli; atlas.

INTRODUCTION

The mammary gland is comprised of parenchymal structures that invade the mammary fat pad. In the mouse, there are five pairs of mammary fat pads located just below the skin, which extend from the thoracic (three pairs) to the inguinal (two pairs) regions of the animal along what is termed the milk or mammary line. Each fat pad has an exterior nipple (1–3) to which the primary epithelial duct is connected to allow the release of milk during lactation (arrows, Figs. 1A and 2A). A lymph node (noted by the asterisk in Figs. 1C and 2B) is located in each of the fat pads (4) and is often used as a landmark when examining histological sections or whole mounts. There is a gradient of differentiation among the glands, with the first thoracic gland being the least differentiated and the fifth inguinal gland the most (5). The description of histology presented in this atlas will use examples of both the third and fourth glands from FVB mice. These extend laterally and dorsally from the milk line in the thoracic (third gland) and abdominal (fourth gland) regions of the mouse and are most easily dissected. Although the features described hold true for other strains of mice, it is important to note that the developmental time points may vary slightly.

The majority of mammary gland development takes place postnatally beginning with the onset of puberty. Prior to birth, there is invasion of the mammary fat pad by a rudimentary ductal structure. This epithelial structure then remains quiescent until approximately 3 weeks of age (Figs. 2A and 3A), when the ovaries begin secreting hormones (3). At this time, the terminal end buds (TEBs,[5] arrows, Figs. 2B and 3A, B) appear and begin the process of ductal elongation, which continues until approximately 10–12 weeks of age, when the TEBs reach the limits

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[5] Abbreviations: TEB, terminal end bud; TUNEL, terminal transferase end-labeling.
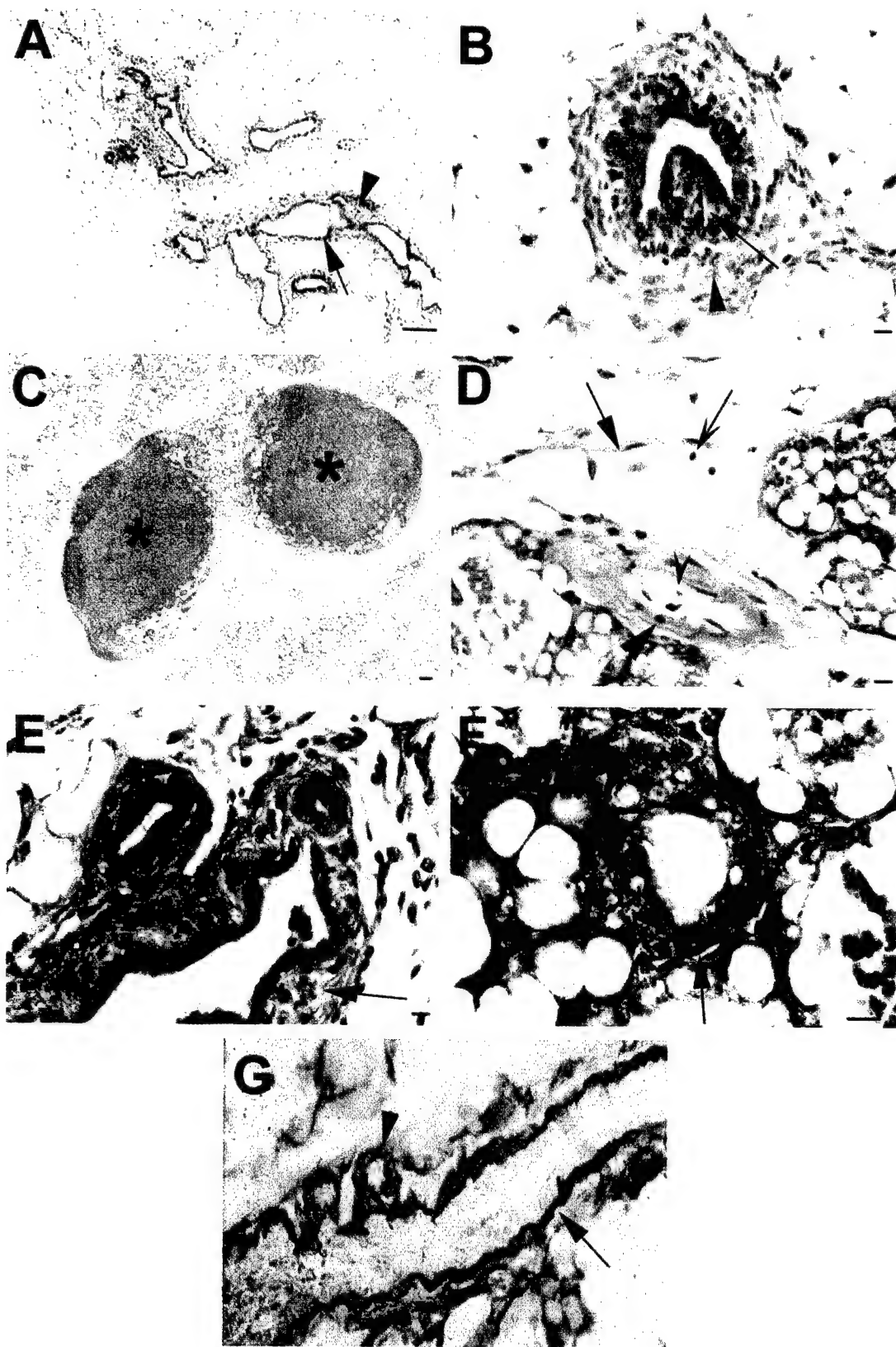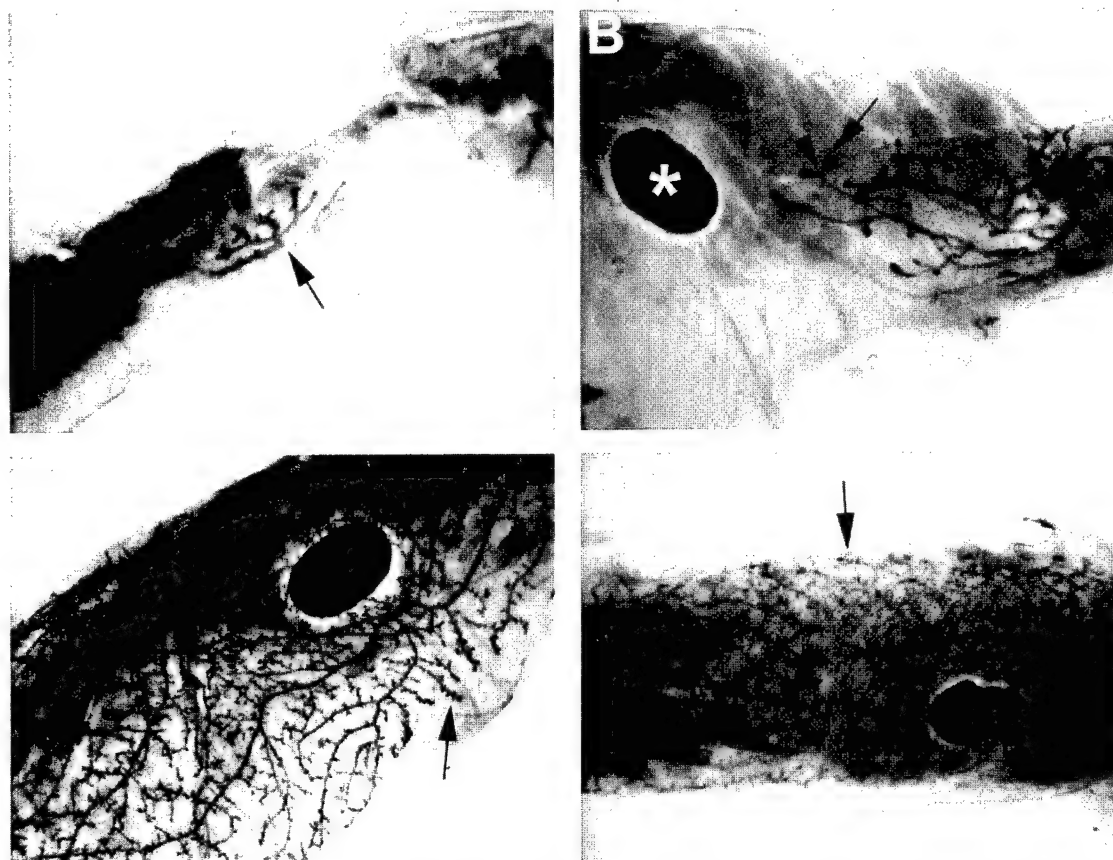
Fig. 2. Whole-mount analysis of mammary glands from FVB mice. The fourth inguinal mammary glands were dissected from female mice at the indicated stages and stained with hematoxylin. (A) Mammary gland from a 3-week virgin. The arrow indicates the nipple region and the primary duct of the epithelial structure. Photographed at 10× magnification. (B) Mammary gland from a 5-week virgin. The lymph node (asterisk) is commonly used as a marker in whole-mount analysis. Ductal growth is occurring as indicated by the TEBs (arrow) and branch points (arrowhead). Photographed at 45× magnification. (C) Mammary gland from a 10-week virgin. Alveolar buds are beginning to form along the ducts (arrow). Photographed at 45× magnification. (D) Mammary gland from day 21 of involution. The ductal tree is similar to that of a prepregnant gland, although some alveolar structures persist (arrow). Photographed at 45× magnification.

of the fat pad and regress (arrow, Fig. 3D) (3,6–8). With the onset of the estrous cycle at puberty, the gland begins to branch (arrow, Fig. 3C) and form alveolar buds (arrow, Fig. 2C; arrowhead, Fig. 3E).

These alveolar buds differentiate completely during pregnancy, when they become capable of milk secretion. At parturition, the alveoli (Fig. 3I) begin copious milk secretion, which continues for about 3 weeks.

Fig. 1. Histological structures of paraffin-embedded mammary glands from FVB mice. Panels A–D were stained with hematoxylin and eosin. Panels E–F were stained with Masson's trichrome. Panel G was immunostained with an anti-laminin antibody. (A) Primary duct and nipple region from a 10-week virgin. The luminal epithelial cells (arrow) are surrounded by a thick stromal layer (arrowhead). (B) A regressing TEB (arrow) found at the edge of the fat pad from a 5-week virgin. The layer of stroma surrounding the TEB is indicated by the arrowhead. (C) The centrally located lymph node present in the mammary gland (asterisk). (D) Blood vessels and lymphatic vessels in the mammary gland. Blood vessels are lined with flattened endothelial cells (thick arrowhead) and contain eosinophilic red blood cells (thin arrowhead). The lymphatic vessels are also lined with flattened epithelial cells (thick arrow) and contain lymphocytes (thin arrow). (E) The blue staining indicates the dense stroma (arrow) surrounding a duct in a 5-week virgin mouse (Masson's trichrome stain). (F) The blue staining indicates the thin stroma (arrow) surrounding an alveolus from an 18-day pregnant mouse (Masson's trichrome stain). (G) Basement membrane surrounding a duct from a 6-day pregnant mouse is visualized by immunostaining with an anti-laminin antibody (arrow). The arrowhead indicates an area of branching where membrane staining is decreased. Magnification bars in panels A and C represent 100 μm. Magnification bars in panels B and DF represent 10 μm. Panel G was photographed at 400× and has been cropped and enlarged, so the magnification is not exact.
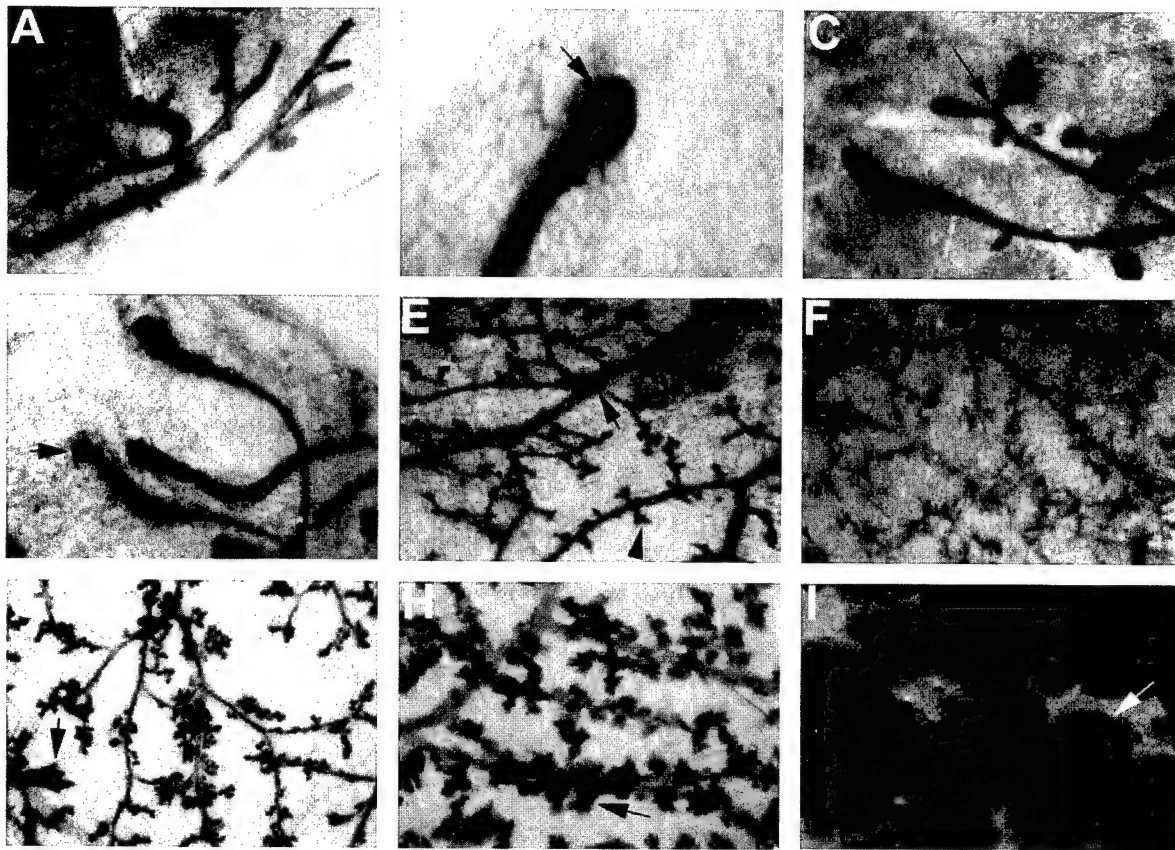
Fig. 3. Whole-mount analysis of mammary gland structures from FVB mice. The fourth inguinal mammary glands were dissected from female mice at the indicated stages, fixed, and stained with hematoxylin. (A) Epithelial structures from a 3-week virgin. TEBs are indicated by the arrow. (B) A TEB (arrow) from a 5-week virgin. (C) Epithelial structures from a 5-week virgin. Bifurcation of the growing duct is indicated by the arrow. (D) Epithelial structures from a 10-week virgin showing a regressing end bud (arrow). (E) Ducts from a 10-week virgin showing both lateral branches (arrow) and alveolar buds (arrowheads). (F) Epithelial structures from a mammary gland at day 21 of involution. (G) Alveolar structures from a mammary gland at day 8 of pregnancy. The alveolar lobules are beginning to develop (arrow). (H) Alveolar structures from a mammary gland at day 12 of pregnancy. The alveoli are continuing to develop (arrow). (I) Alveolar structures from a mammary gland at day 18 of pregnancy. The arrow indicates the alveoli that have filled the majority of the fat pad. Although not visible, there are still areas of fat within the gland that will not disappear until lactation. All panels were photographed at 45× magnification. Panels A–D have been cropped and enlarged to show the structures of interest, so magnification is not exact.

At weaning, the gland begins a process of tissue remodeling which involves apoptosis of mammary epithelial cells (9–11). This process is reversible during the first 2 days as pups can be returned to the mother and lactation resumes (12). After 2 days postweaning, lactation cannot be reinitiated and the gland regresses back to a nearly prepregnant state (compare Fig. 3E, F). This process of involution (Figs. 7 and 8) takes approximately 2 weeks to complete (11). At this time, the gland is ready to initiate another cycle of pregnancy, lactation, and involution.

EMBRYONIC DEVELOPMENT

The mammary gland first appears embryonically as an epithelial bud that penetrates the underlying mesenchyme (see Ref. 2 for a review of embryonic development). This begins at embryonic day 10–11 when the mammary streak appears as a line extending from the anterior to the posterior limb bud due to enlargement of a single-layered ectoderm. By day 12, the mammary epithelium has the shape of a lens and consists of several cell layers. This mammary

rudiment becomes bulb-shaped by day 14 of embryonic development and elongates beginning at day 16 to form a mammary sprout due to rapid proliferation of the epithelial cells. At day 17, this sprout invades the underlying mammary fat pad precursor tissue and continues to grow until birth to form a rudimentary ductal tree with several branching ducts. In the male mouse of some strains, mammary development is identical to that of the female until day 13, when the testes begin to produce androgens. In response to androgens, the mammary epithelium of the male mouse is decreased in volume and becomes irregular in shape. On day 15, the epithelium connecting the rudimentary gland with the epidermis becomes narrow and detaches, leaving the mammary rudiment within the fat pad.

After embryonic development, the gland remains inactive until approximately 3 weeks of age when the ovaries begin to secrete hormones (3). Postnatal growth of the mammary gland occurs in two major phases: ductal growth, which is initiated with puberty, and alveolar differentiation, which occurs primarily during pregnancy. Ductal growth involves lengthening and branching of the epithelium, which ultimately extends to the limits of the fat pad. Alveolar differentiation results in the terminal differentiation of the gland, which involves the formation of the milk-secreting units and culminates in lactation.

POSTNATAL DEVELOPMENT

At birth, the mammary gland consists of the epithelial cords and the stroma, which includes connective tissue, fibroblasts, and the mammary fat pad. The parenchyma at this stage is rudimentary and consists of a small ductal tree (Figs. 2A and 3A) (13,14). Each branch is composed of a single layer of epithelial cells surrounding a central lumen; the cells bordering the lumen are referred to as luminal epithelial cells (arrows, Figs. 1A and 4C). Epithelial cells can be identified immunohistochemically by the presence of keratins 8, 11, 14, 20, and 22 in the mouse (15). Beneath the epithelial cells, the myoepithelial cells (arrow, Fig. 4E; thin arrowhead, Fig. 4C) form a basal layer that rests on a laminin-containing basement membrane (arrow, Fig. 1G), which separates the parenchymal and stromal compartments. The myoepithelial cells can be identified by immunohistochemical staining with anti-smooth muscle actin antibody (Figs. 4E, 6E, and 8D) (16). These cells vary in

appearance throughout development from a sheath around the epithelial cells during ductal development (Fig. 4E) and involution (Fig. 8D) to a discontinuous layer of cells whose dendrites circle the alveoli during lactation (Fig. 6E) (3,17). The myoepithelial cells are contractile and are responsible for the movement of milk out of the alveoli and down the ducts during lactation (15,18,19). These cells are also responsible for the secretion of basement membrane components during all developmental stages (3,17,19). The connective tissue stroma is thick and dense around these epithelial structures and consists of eosinophilic fibrous connective tissue and fibroblasts (thin arrow, Fig. 4C), visualized using Masson's trichrome staining (Fig. 1E, F). The density of this stroma varies with development. The stroma is much thinner around small ductules and alveoli than ducts (compare Fig. 1E, F).

The basement membrane is composed of a thin layer of proteins which lies next to the basal surface of the myoepithelial cells of the ductal structures. It consists of an organized network of proteins and proteoglycans which are locally secreted by the myoepithelial cells and include fibronectin, laminin, type IV collagen, and heparan sulfate proteoglycans (20–22). This layer can be easily visualized by immunohistochemical staining for laminin (arrow, Fig. 1G). Maintenance of the ductal structures requires that this membrane be intact since it not only provides structural support for the cells, but also influences epithelial cell shape, polarity, growth, and responsiveness to hormones (23). Epithelial and myoepithelial cells control their interactions with the basement membrane by remodeling the immediate environment through secretion of basement membrane-degrading proteases and protease inhibitors (24). Rearrangement of this layer is important for normal development, differentiation, and function of the mammary gland (25).

At the early stages of development, the stromal compartment of the gland is filled with large adipocytes (Fig. 4C, "a"; Fig. 8C, asterisk), which in the mouse can be either unilocular (black asterisk, Fig. 6A) or multilocular (white asterisk, Fig. 6A) (26). Multilocular fat is believed to be immature and is often observed during lactation, when the fat is being mobilized for use, and during involution, when the adipocytes are reappearing. In addition to being present in the dense stroma, fibroblasts are interspersed with the adipocytes. Also present in the gland are blood vessels and lymphatics (Fig. 1D). Blood vessels

Fig. 4. Histological features of the mammary gland during pubertal and postpubertal development in the FVB mouse. Panels A–D were stained with hematoxylin and eosin. Panel E was immunostained with an anti-smooth muscle actin antibody to visualize myoepithelial cells. (A) A TEB from a 5-week virgin. The connective tissue proximal to the cap cells of the TEB is absent (arrow). The absence of the lower left corner of tissue is due to the fact that this end bud was cut from a whole mount and is not a sectioning artifact. The thin arrow indicates initiation of the hollowing of the TEB that results in the lumen of the duct. At the trailing edge of the TEB, cells are differentiating, and the stroma is forming around the ducts (arrowhead). (B) Higher magnification of a TEB from a 5-week virgin. At the leading edge of the TEB, cap cells are undergoing extensive proliferation (arrowhead). The epithelial cells adjacent to the cap cells are also increasing in number (white asterisk). A layer of body cells (arrow) is undergoing apoptosis to form the lumen of the duct (black asterisk). Stroma is absent at the growing tip of the end bud (thin arrow). (C) A duct from a 5-week virgin. A single layer of epithelial cells (arrow) lines the lumen of the duct (asterisk) and is surrounded by a single layer of myoepithelial cells (thin arrowhead) and a thick layer of dense stroma (arrowhead) consisting of connective tissue and fibroblasts (thin arrow). At this stage, the majority of the gland is filled with adipocytes (a). (D) An alveolar bud from a 10-week virgin. (E) Myoepithelial cells, visualized by immunostaining with an anti-smooth muscle actin antibody, form a layer around a duct in a 5-week virgin (arrow). Magnification bar in panel A represents 100 μm. Magnification bars in panels B–D represent 10 μm. The photo in panel E was taken at 200× and was cropped and enlarged, so the magnification is not exact.

(thick arrowhead, Fig. 1D) are easily recognized in hematoxylin and eosin-stained sections by the presence of eosinophilic, anucleate red blood cells in the lumen (thin arrowhead, Fig. 1D) and by the layer of flattened endothelial cells, which contrast with the cuboidal epithelium of the ducts (arrow, Fig. 4C). Blood vessels are present throughout mammary gland development, with highest numbers during pregnancy and lactation (27). The lymph node (asterisk, Fig. 1C) is also easily recognized by the dense region of dark blue-staining lymphocytes. Lymphocytes (thin arrow, Fig. 1D) are also present in lymphatic vessels (thick arrow, Fig. 1D), which are lined by a thin layer of flattened endothelial cells similar to those observed in blood vessels. Lymphatics may look like large adipocytes if lymphocytes are not present. Often the blood vessels and lymphatics are observed side by side in hematoxylin and eosin-stained sections as observed in Fig. 1D.

Puberty and Postpuberty

The period of most rapid ductal growth occurs during puberty from approximately 3–6 weeks of age in the mouse. In rodents the glands do not regress during prepuberty, but maintain a small ductal tree, which initiates rapid growth at the onset of puberty (Figs. 2A and 3A) (3). During pubertal and postpubertal growth, the ducts lengthen and branch to form secondary and tertiary (Fig. 2B) ducts that ultimately extend to fill the mammary fat pad by approximately 3 months of age (Fig. 2C).

The primary duct (arrow, Fig. 1A) is large and consists of a layer of epithelial cells surrounded by a thick layer of dense stroma (arrowhead, Fig. 1A) (3). This large lumen functions as a reservoir for milk during lactation and as the connection to the exterior nipple. This duct can be identified based on its proximity to the nipple and its thick connective tissue sheath. In addition, while secretion occurs in the alveolar structures, there is no secretion in the primary and secondary ducts (M. Richert, unpublished observation). The secondary and tertiary ductal structures are composed of a single layer of cuboidal epithelial cells surrounding a central lumen (arrow, Fig. 4C) (3,28). A layer of myoepithelial cells, which sits on the basement membrane, lies basal to the epithelial cells (arrow, Fig. 4E). Surrounding the basement membrane is a thin layer of dense stroma comprised of connective tissue and fibroblasts (arrowhead, Fig. 4C).

The primary epithelial structure in rodent mammary glands at the start of ductal growth is the terminal end bud (TEB), which first appears at approximately 3 weeks of age (arrows, Fig. 2B, Fig. 3A, B, and Fig. 4A, B) (4,14,29). These structures are the sites of ductal elongation and branching. TEBs are bulbous structures located at the tips of the growing ducts and they represent the sites of highest proliferation in the gland (3,4). TEBs consist of multiple layers of epithelium with an outer layer of undifferentiated, pluripotent stem cells called cap cells which sit on the basal lamina (arrowhead, Fig. 4B) (3,17). Surrounding these cap cells, the connective tissue stroma is absent (arrow, Fig. 4A; thin arrow, Fig. 4B) (3,17). Cap cells have the highest proliferative rate of any cell population in the mammary gland and eventually give rise to the myoepithelial cells of the ducts (4). These cells are also capable of migrating into the underlying epithelial layers and becoming luminal epithelial cells (3,17). Just beneath the layer of cap cells, proliferation of the epithelial cells within the TEB results in increased numbers of cells (white asterisk, Fig. 4B), which eventually become the ductal epithelial cells (3,17). TUNEL staining has demonstrated that the innermost layers of body cells (arrow, Fig. 4B) are undergoing apoptosis (6), presumably to form the lumen of the duct (arrow, Fig. 3B; thin arrow, Fig. 4A; black asterisk, Fig. 4B). During the growth phase, the proliferative edge of the TEB is not surrounded by connective tissue or myoepithelial cells, and therefore proliferation and cell migration result in invasion into the fat pad and elongation of the duct. These TEBs can split, resulting in branching (arrowhead, Fig. 2B) (30,31) and formation of alveolar buds (arrow, Fig. 2C; arrowhead, Fig. 3E) during the differentiation process. Cell division drops significantly in the cells at the trailing edge of the end bud as they differentiate into ductal and myoepithelial cells (6). As the cells differentiate at the trailing edge, the myoepithelial cells lay down the basement membrane and the stroma is formed by fibroblasts surrounding the ducts (arrowhead, Fig. 4A) (17). When the TEBs reach the connective tissue surrounding another epithelial structure or the capsule at the edge of the fat pad, they regress to form terminal ducts (arrow, Fig. 3D) (3,6–8). The regressing TEB (arrow, Fig. 1B) becomes encased in basement membrane and stroma (arrowhead, Fig. 1B) and is hollowed to form a single layer of epithelial cells surrounded by myoepithelial cells and basement membrane.

At 10–12 weeks of age, the majority of the TEBs have reached the edge of the fat pad and have re-

gressed. The development of lateral and alveolar buds is initiated in the postpubertal gland in response to the cyclic secretion of ovarian hormones with each estrous cycle (32; see also Schedin et al., this issue). The lateral buds can form branches (arrow, Fig. 3E) or cleave to form alveolar buds (arrowhead, Figs. 3E and 4D). Lateral buds that will form branches have a layer of cap cells at the growing tip similar to TEBs (3). As branching morphogenesis occurs, the connective tissue within the extracellular matrix is broken down to allow growth of the epithelial structures (arrowhead, Fig. 1G) (3). The alveolar buds subdivide to form rudimentary alveolar structures as postpubertal growth continues. These structures are composed of a single layer of epithelial cells enveloping a circular hollow center. Progression of these alveolar buds into fully differentiated units capable of milk secretion occurs only during pregnancy-induced growth of the mammary gland, although expression of milk proteins can be observed during phases of the estrous cycle as the gland exhibits a cycle of mild proliferation, differentiation, and involution (33–35; see also Schedin et al., this issue).

Pregnancy and Lactation

The peak of mammary differentiation occurs during the 19–21 days of pregnancy and culminates with formation of alveoli and a fully lactating gland at parturition (36). Initial pregnancy-induced mammary growth involves massive proliferation of ductal branches (Fig. 3G and arrow, Fig. 5A) and formation of alveolar buds like those observed during postpubertal development (Fig. 3G and Fig. 5A, D, G) (36). The epithelial to adipocyte ratio increases and capillaries are found within the connective tissue surrounding each individual alveolus (arrow, Fig. 5I). During the second half of pregnancy, the alveolar buds progressively cleave and differentiate into individual alveoli that will ultimately become milk-secreting lobules during lactation (arrows, Fig. 3H, I, and Fig. 5B, C, E, F, H, I). This later stage of differentiation is commonly referred to as the lobuloalveolar phase of mammary growth. By late pregnancy, the alveoli fill the majority of the fat pad (Figs. 3I and 5C), at which time the gland is capable of milk production. Leakiness of the cell junctions results in the presence of protein in the lumen, which stains purple/pink in hematoxylin and eosin-stained sections (asterisk, Fig. 5F) (37). By day 18 of pregnancy, the alveolar epithelial cells are producing milk proteins and lipid (arrow,

Fig. 5F) in preparation for lactation. The epithelial cells are enlarged due to accumulation of lipid (Fig. 5I) (38). The myoepithelial cells still surround the alveoli, but are less continuous, so that epithelial cells may come into direct contact with the basement membrane; this contact may be critical to full differentiation and milk secretion (20,21,34,39,40). The amount of stroma in the gland is greatly decreased at this time, allowing more contact of the epithelium with adipocytes (arrowhead, Fig. 5F) (26,41). Fully developed alveolar structures remain until the completion of lactation.

Approximately 1 day prior to parturition, the epithelial cells accumulate rough endoplasmic reticulum and Golgi and secretory vesicles and begin secreting milk proteins and lipids (38,42). In alveoli that are engorged with secretions after the onset of lactation, the shape of the luminal epithelium changes from cuboidal or pyramidal to flattened and the presence of milk fat globules within the cells is easily observed (Fig. 6C, D). The lumen becomes filled with milk, which is observed as a pink/purple stain with circles of milk fat visible in hematoxylin and eosin-stained sections (asterisk, Fig. 6B, and Fig. 6C, D). Approximately 30% of the gland is still filled with adipocytes (Fig. 6A). A portion of these adipocytes are multilocular (white asterisk, Fig. 6A) possibly due to the mobilization of the fat for use by the epithelial cells. Red blood cells, indicating blood vessels, and a thin layer of connective tissue are still visible between the alveoli (arrows, Fig. 6C, D). Myoepithelial cells form a discontinuous sheath around each alveolus (arrow, Fig. 6E) and contract their dendritic processes in response to oxytocin to force the milk out of the alveolus and into the ducts (15,18,19). As lactation becomes established, the fat in the adipocytes is metabolized and the alveoli expand to completely fill the gland (Fig. 6B) (43). Flattened epithelial cells in the process of secreting milk fat droplets are easily visible at higher magnification (arrowhead, Fig. 6D). The process of lactation continues for approximately 3 weeks until the pups are weaned.

Involution

After weaning, the gland goes through a process of death and remodeling termed involution. This process is initiated by milk stasis once milk removal has ceased (45). The process of involution described here follows forced weaning of the pups. The pups were allowed to suckle for 8 days postpartum and then
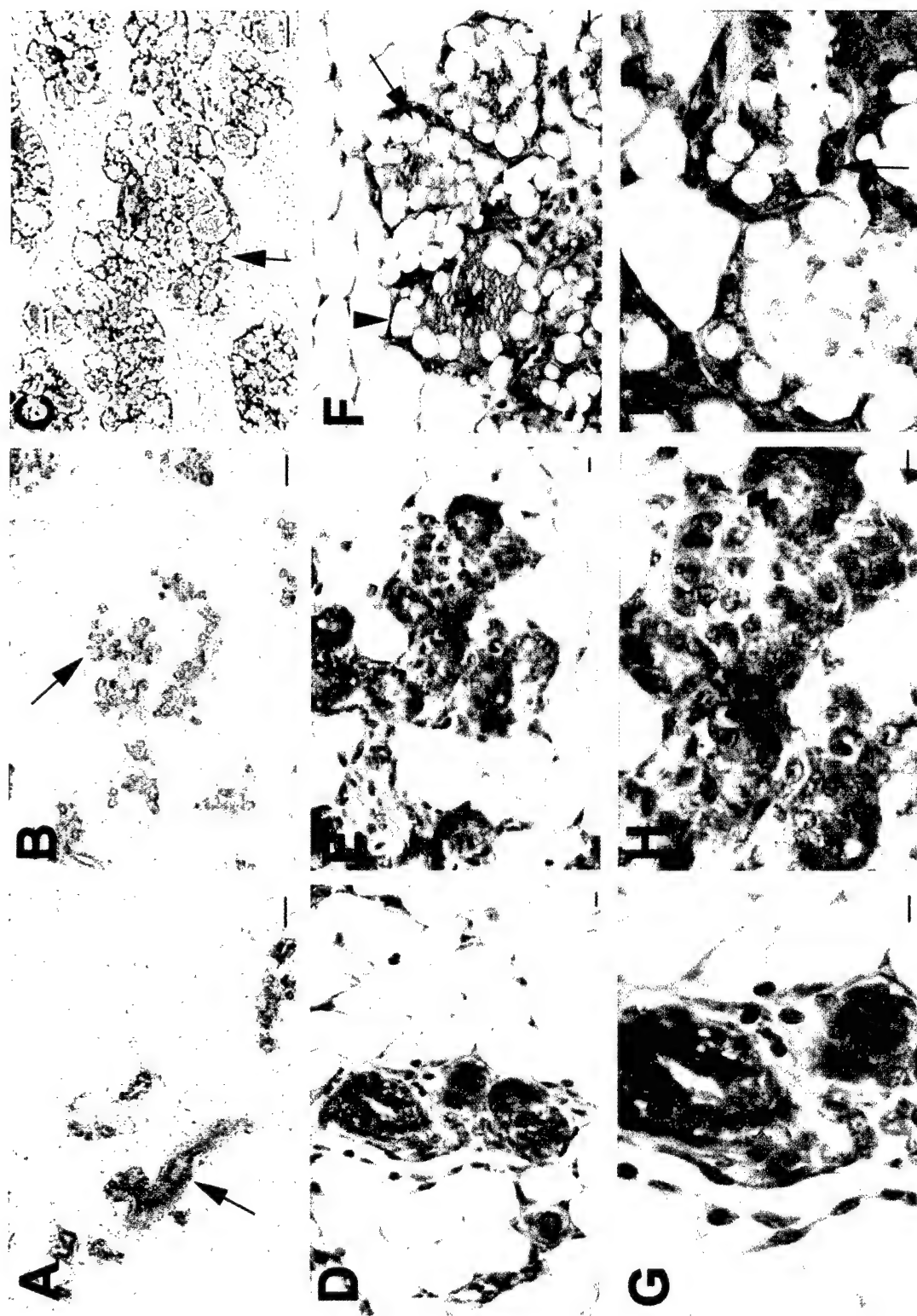
Fig. 5. Histological features of the mammary gland during pregnancy in the FVB mouse. All sections have been stained with hematoxylin and eosin. (A, D, G) Mammary gland from a day 6 pregnant mouse. Ducts are visible (arrow, A) and alveolar lobules are beginning to develop. Panels D and G are higher magnification views of the developing alveoli. (B, E, H) Mammary gland from a day 12 pregnant mouse. The alveoli continue to develop (arrow, B) throughout pregnancy. Panels E and H are higher magnification views of the developing alveoli. (C, F, I) Mammary gland from a day 18 pregnancy mouse. The alveoli have continued to develop and have filled the majority of the fat pad (arrow, C). The epithelial cells have begun to produce milk proteins and lipid (arrow, F). The luminal spaces are filled with a substance (asterisk, F) that may arise from the interstitial space due to the open tight junctions. Because of the decreased stroma during this stage, the epithelial cells have more contact with the adipocytes (arrowhead, F). The individual alveoli are surrounded by blood vessels (arrow, I). Magnification bars in panels A–C represent 100 μm. Magnification bars in panels D–I represent 10 μm.
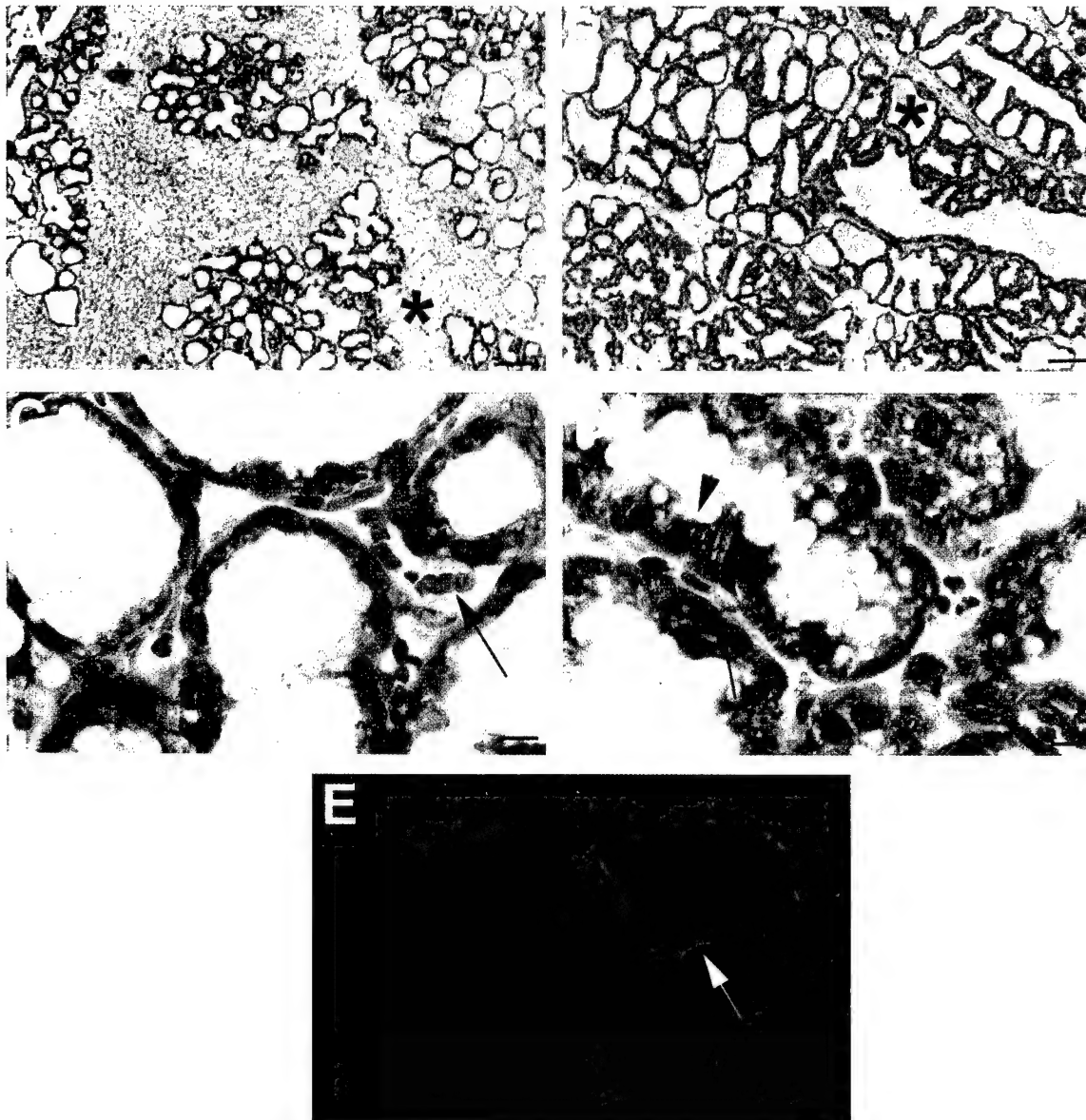
Fig. 6. Histological features of the mammary gland during lactation in the FVB mouse. Panels A–D were stained with hematoxylin and eosin. Panel E was immunostained with an anti-smooth muscle actin antibody to visualize myoepithelial cells. (A, C) Mammary gland structures at day 2 of lactation. Secretory alveolar structures are present in the mammary gland and are surrounded by both unilocular fat cells (black asterisk, A) and multilocular fat cells (white asterisk, A). Panel C shows a higher magnification of the secretory structures. Alveolar capillaries are abundant as seen by the red blood cells (arrow, C). (B, D) Mammary gland structures at day 9 of lactation. The secretory alveolar structures have filled the fat pad and milk can be identified in the luminal spaces (asterisk, B). Panel D shows a higher magnification of the secretory structures. The secretion of milk lipid droplets by epithelial cells results in a vacuolated, disorganized appearance at the apical surface (arrowhead, D). (E) Myoepithelial cells, visualized by immunostaining with anti-smooth muscle actin antibody, form a discontinuous layer around the alveoli (arrow). Magnification bars in panels A and B represent 100 μm. Magnification bars in panels C and D represent 10 μm. The photo in panel E was taken at 200$\times$ and was cropped and enlarged, so the magnification is not exact.

were removed from the cage. The mothers were sacrificed at various time points. This process is somewhat artificial as natural weaning occurs much more slowly. Forced weaning is often chosen as a model for involution because it is more controlled than natural weaning and allows for more precise timing of structural changes. The process of natural weaning has not been studied to the same extent as forced weaning, but it is likely that the processes and histological features occur in a similar manner, but are extended over a longer period of time.

Initially, involution is reversible and lactation can be reinitiated upon suckling (12,46). Histologically the gland appears similar to what is observed during lactation; aside from flattening of the epithelium due to engorgement of the alveoli with milk, there are no major morphological changes during this reversible stage. After 2 days, the gland begins the irreversible sequence of cell death and remodeling. During this process, the secretory epithelial cells of the alveoli begin to undergo apoptosis, as measured by nick-end DNA labeling, with a peak at day 4 of involution (47). The percentage of apoptotic epithelial cells as well as the peak day may vary between different mouse strains and with the choice of detection method. These apoptotic cells can be cleared either by neighboring epithelial cells or by invading macrophages (9,44,48–50). At day 2 of involution, the alveoli appear to be relatively intact (arrowhead, Fig. 7A) with some apoptotic bodies visible in the luminal space (arrows, Figs. 7A and 8A) along with lipid and a substance that stains similarly to milk in hematoxylin and eosin-stained sections. The epithelial structures still appear organized (thin arrow, Fig. 8A). As the alveolar epithelial cells begin to die, the alveoli collapse into clusters of epithelial cells. This occurs by day 4 of involution (Figs. 7B and 8B). At this time, the adipocytes appear to be refilling, as a high percentage of multilocular fat cells is visible. The epithelium appears very disorganized (arrowhead, Fig. 8B) although ducts are readily observed and dense stroma is visible around the ducts. Through day 4 alveolar structures are still present (Figs. 7B and 8B). The area comprised of adipocytes continues to increase as epithelium decreases. The stroma also increases around the clusters of collapsing alveoli.

At day 6 of involution (Figs. 7C and 8C), all of the alveoli have collapsed and both epithelium and stroma are being rearranged (11). The timing of basement membrane remodeling detected by histological examination is consistent with the changes in expression of proteases and inhibitors described in biochemical studies (51). The majority of cell death has already occurred, although apoptotic bodies are still visible through day 8 and even out to day 14 (arrowhead, Fig. 7E). At this time, ducts are visible along with what appear to be small, disorganized clusters of epithelial cells and increased stroma (Fig. 7D). A sheath of myoepithelial cells surrounds the involuting alveoli (arrow, Fig. 8D). This process continues until the gland is completely remodeled and resembles the prepregnant mature gland by day 21 of involution (Figs. 2D, 3F, and 7F). The remodeled gland appears somewhat more differentiated than a gland from a 10-week virgin animal, with the presence of some alveoli throughout the gland (arrows, Figs. 2D and 7F). The epithelial structures at this stage consist of a single layer of epithelial cells. A meshwork of myoepithelial cells resting on the basement membrane surrounds all of the epithelial structures. A thick layer of dense stroma surrounds the ducts, while a more discontinuous layer encases the alveolar structures. As in virgin mice, the majority of the gland is comprised of unilocular adipocytes.

This atlas was composed to present a brief description of mouse mammary gland morphology and histology throughout each developmental stage and to illustrate examples of how to identify specific structures of the gland in order to understand the developmental changes observed in mouse models of mammary gland development. As mentioned earlier, this is a description of the cyle of mammary development and involution in FVB mice. There may be strain-specific differences in this process, but the general histological features are believed to apply to all strains of mice.
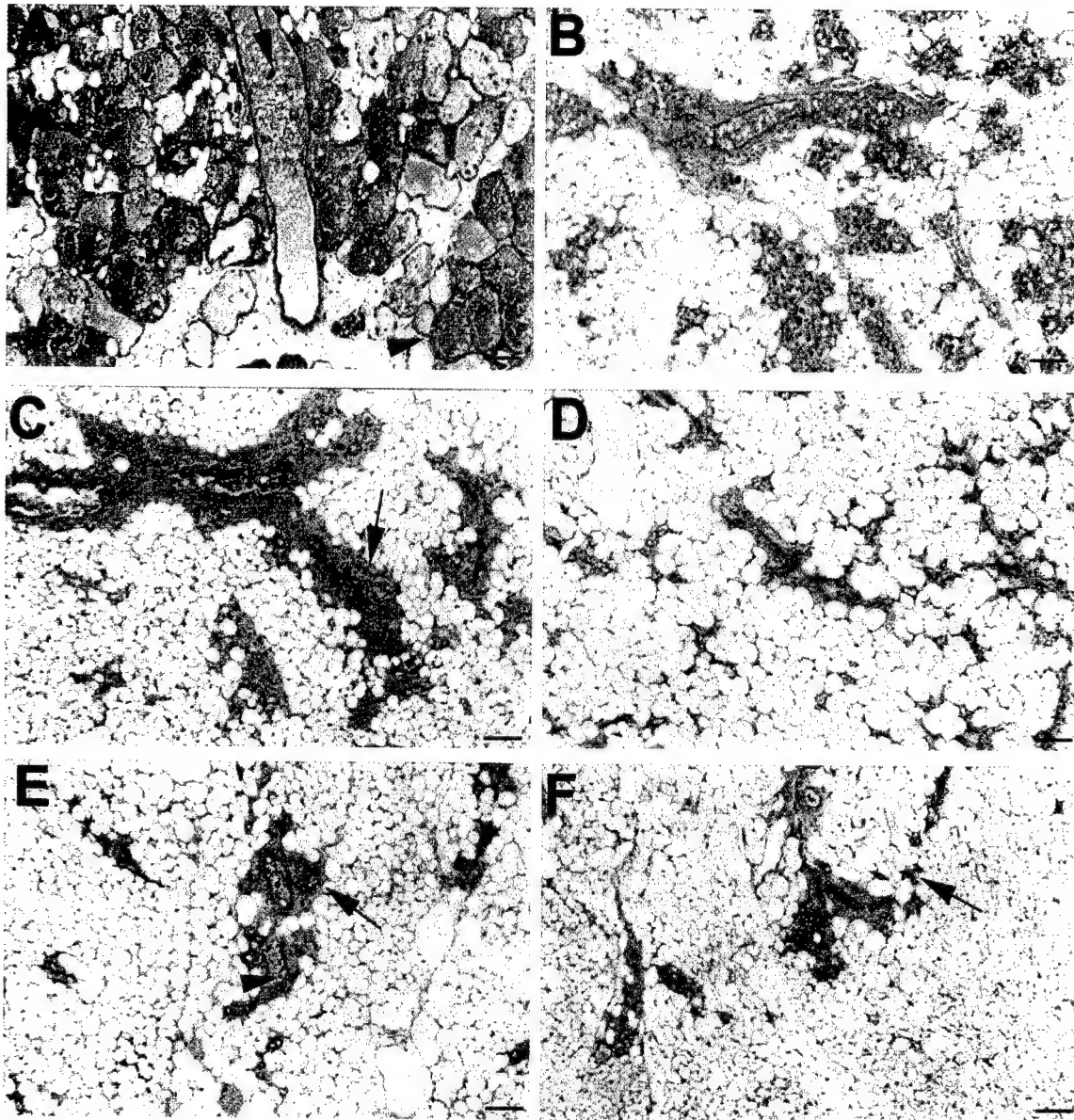
ACKNOWLEDGMENTS

Fig. 7. Histological features of mammary gland involution in the FVB mouse. All panels were stained with hematoxylin and eosin. (A) Mammary gland structures at day 2 of involution. The alveoli are still intact (arrowhead); however, apoptotic cells can be identified in the luminal spaces (arrow). (B) Mammary gland structures at day 4 of involution. The alveoli have begun to collapse, although some alveolar structures can still be identified. (C) Mammary gland structures at day 6 of involution. the alveoli have collapsed and the stroma has increased around the collapsed structures (arrow). (D) Mammary gland structures at day 8 of involution. Ducts and small clusters of epithelial cells are visible. (E) Mammary gland structures at day 14 of involution. The mammary gland has been remodeled, although apoptotic bodies can still be seen in the luminal spaces (arrowhead). Ducts are visible and are surrounded by thick stroma (arrow). (F) Mammary gland structures at day 21 of involution. The mammary gland has been completely remodeled and resembles a prepregnant gland, although alveolar structures can be identified (arrow). Magnification bars in all panels represent 100 μm.
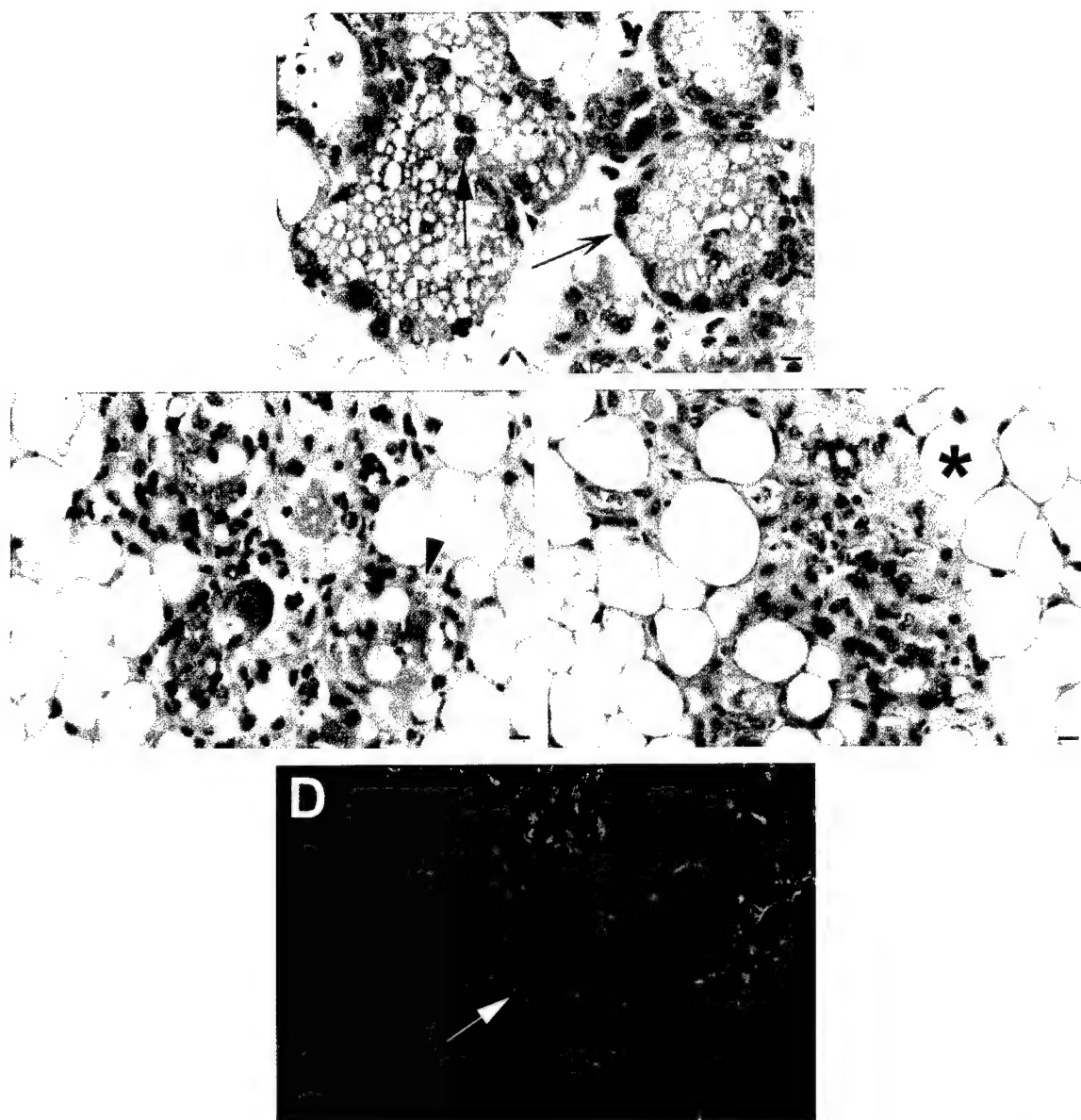
Fig. 8. Higher magnification view of the involuting structures seen in Fig. 7. Panels A–C were stained with hematoxylin and eosin. Panel D was immunostained with an anti-smooth muscle actin antibody to visualize myoepithelial cells. (A) Mammary gland structures at day 2 of involution. The alveolar structures are still intact and the epithelial cells are still organized (thin arrow). Apoptotic bodies can be identified in the luminal spaces of the alveolar structures (arrow). (B) Mammary gland structures at day 4 of involution. The epithelium is becoming more disorganized (arrowhead). (C) Mammary gland structures at day 6 of involution. The alveoli are very disorganized and the adipocytes are repopulating the gland (asterisk). (D) Myoepithelial cells can be identified by immunostaining with an anti-smooth muscle actin antibody as a sheath surrounding epithelial structures. Magnification bars in panels A–C represent 10 μm. The photo in panel D was taken at 200× and was cropped and enlarged, so the magnification is not exact.

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Mammary Gland Involution Is Delayed by Activated Akt in Transgenic Mice

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Activation of the antiapoptotic protein kinase Akt is induced by a number of growth factors that regulate mammary gland development. Akt is expressed during mammary gland development, and expression decreases at the onset of involution. To address Akt actions in mammary gland development, transgenic mice were generated expressing constitutively active Akt in the mammary gland under the control of the mouse mammary tumor virus (MMTV) promoter. Analysis of mammary glands from these mice reveals a delay in both involution and the onset of apoptosis. Expression of tissue inhibitor of metalloproteinase-1 (TIMP-1), an inhibitor of matrix metalloproteinases (MMPs), is prolonged and increased in the transgenic mice, suggesting that disruption of the MMP:TIMP ratio may contribute to the delayed mammary gland involution observed in the transgenic mice. (Molecular Endocrinology 15: 867–881, 2001)

INTRODUCTION

Mammary gland involution is characterized by a decrease in milk protein synthesis, extensive apoptosis of the secretory alveolar cells, and structural remodeling of the gland, which requires the activation of matrix metalloproteinases (MMPs) and the inactivation of their inhibitors, the tissue inhibitor of metalloproteinases (TIMPs) (1–6). It has been reported that 50–80% of the mammary epithelial cells in mice that are present during lactation undergo apoptosis during involution (2). The number of apoptotic cells during involution peaks at 3–4 days post weaning, and remodeling is complete by 6 to 8 days post weaning (1, 7, 8). Mammary gland involution provides an excellent *in vivo* model for examining mechanisms regulating apoptosis due to the extensive apoptosis of the epithelial

cells and the accessibility of the mouse mammary gland.

The use of mouse genetic models has been critical for gaining insight into the molecular processes of apoptosis during mammary gland involution. A conditional knockout of signal transducer and activator of transcription-3 (STAT3) in the mammary gland results in delayed involution and decreased epithelial cell apoptosis, suggesting that STAT3 is critical for the normal process of apoptosis during involution (9). Transgenic mice overexpressing insulin-like growth factor-I (IGF-I) and insulin-like growth factor binding protein 3 (IGFBP-3) in the mammary gland also exhibit delayed involution and decreased apoptosis (10). IGF-I has been shown to be involved in both proliferation and suppression of apoptosis, and IGFBP-3 may be involved in facilitating the actions of IGF-I in the mammary gland. In addition, mice overexpressing the antiapoptotic protein Bcl-2 in the mammary gland show an inhibition of alveolar cell apoptosis, although the collapse of alveolar structures is not delayed (11). These observations suggest a role for these proteins in regulating mammary gland involution. Finally, the expression patterns of a number of apoptosis-related proteins, including Bcl-x, Bax, p53, transforming growth factor β1 (TGF-β1), and sulfated glycoprotein 2 (SGP-2), are regulated during involution (1, 12, 13). The further identification of proteins that regulate the apoptotic process during involution is important for understanding both the regulation of normal mammary gland development and the process of apoptosis in physiological systems.

Akt, also referred to as PKB (protein kinase B) and RAC (related to A and C kinases), is a serine/threonine protein kinase that has been shown to suppress apoptosis in a number of systems. c-Akt (PKBα, RACα) was identified as the cellular homolog of the retroviral oncogene, v-akt (14, 15). In addition to c-Akt (now known as Akt1), two other members of the Akt family have been identified, Akt2 (PKBβ, RACβ) and Akt3 (PKBγ, RACγ) (15, 16). The three members of this family are all serine/threonine protein kinases that share similar structural features, including a pleckstrin

homology (PH) domain in the amino terminus and two regulatory phosphorylation sites, Thr308 and Ser473 in Akt1, which are critical for activation (17). Numerous studies have shown that Akt is activated in a phosphatidylinositol 3′-kinase (PI3K)-dependent manner (18, 19). The activation of PI3K by growth factor receptors results in the generation of 3′-phosphoinositides, which recruit Akt to the plasma membrane by means of its PH domain binding to these membrane-associated phospholipids (20). Akt is then phosphorylated by phosphatidylinositol-dependent kinase 1 (PDK1), which has been shown to phosphorylate Thr308 (21, 22). Phosphorylation of Ser473 is also required for catalytic activation of Akt, and it has been suggested that this represents an autophosphorylation event (23), although other kinases may also phosphorylate this site. Localization to the membrane appears to be critical for Akt activation because modifications that target Akt to the membrane, such as the gag sequence found in v-Akt (24) or fusion of a myristoylation sequence from src-like kinases to the amino terminus of Akt (25), result in the constitutive activation of the kinase. Activation of Akt results in the suppression of apoptosis induced by a number of stimuli including growth factor withdrawal, detachment from extracellular matrix, UV irradiation, cell cycle discordance, and activation of Fas (25–31). In addition, expression of gag-Akt, a constitutively active form of Akt, in thymocytes of transgenic mice prevents apoptosis in response to various stimuli (32).

Akt activation can be induced by a number of growth factors, including epidermal growth factor (EGF) (30), IGF-I (17, 33), estrogen (34), and hepatocyte growth factor/scatter factor (35), which all regulate mammary gland development (36–40). The purpose of this study was to examine the role of Akt as a regulator of apoptosis during mammary gland involution. A constitutively active mutant of Akt, which contains a myristoylation sequence fused to the amino terminus of Akt (myr-Akt), was expressed in mammary glands of transgenic mice using the mouse mammary tumor virus (MMTV) promoter present in the long terminal repeat of MMTV. We demonstrate that the expression of constitutively active Akt in the mammary gland results in delayed involution and a delay in the onset of apoptosis, suggesting that Akt may be involved in regulating normal mammary gland involution.

RESULTS

Expression of Akt Is Decreased during Early Involution

Apoptosis of the secretory epithelial cells during mammary gland involution peaks by day 4 of involution and then decreases (7). Therefore, we hypothesized that Akt levels would decrease early during involution to allow apoptosis to occur. To test this hypothesis, we examined the pattern of Akt expression and activation during mam-

mary gland involution. Pregnant FVB female mice gave birth, and the litters were normalized to eight pups each. The pups were allowed to suckle for 9 days to ensure the full establishment of lactation after which time the pups were removed. Forced weaning at day 9 was used in these studies because the onset of involution can be more closely controlled than in natural weaning. The fourth inguinal mammary glands were removed on day 9 of lactation, and days 2, 4, 6, 8, 10, and 14 of involution, and total Akt protein levels were determined by immunoblotting. As shown in Fig. 1A, Akt expression levels were high during lactation, but they decreased dramatically by day 2 of involution. The antibody used in this study does not distinguish between Akt1 and Akt2. The immunoblot was reprobed with an antibody to actin to determine whether the observed decrease in protein expression was a result of milk accumulation that normally occurs early in involution. Although actin levels also decrease at involution day 2, normalization of Akt levels to actin levels indicates that Akt expression is decreased after lactation and remains low throughout involution (Fig. 1B). These data are consistent with results published by Chodosh et al. (41), in which Northern blot analysis shows highest levels of expression of Akt during lactation, which then decrease by day 2 and remain low through day 7 of involution.

Akt activation during involution was determined by examining the ability of immunoprecipitated Akt to phosphorylate a known substrate, glycogen synthase kinase-3 (GSK-3), in vitro (42, 43). The pattern of Akt activity is similar to the pattern of expression; Akt activity is high during lactation followed by a decrease in activity by day 2 of involution and remains low throughout involution (Fig. 1C). These data suggest that Akt may be involved in suppression of apoptosis during lactation and that a decrease of Akt expression may be critical for apoptosis to occur during involution.

To determine localization of Akt in the mammary gland, immunofluorescence was performed on paraffin-embedded sections from lactating glands using an anti-Akt1 specific antibody. As shown in Fig. 1D, Akt1 is expressed in epithelial cells surrounding the luminal spaces as visualized by the red staining. The luminal spaces are visualized by Oregon-Green 488-conjugated wheat germ agglutinin (WGA), which binds to mucins on the apical surfaces of epithelial cells (44). Blue staining represents the 4,6-diamidino-2-phenylindole (DAPI)-stained nuclei. No staining is observed in the negative control slides that received only the secondary antibody (Fig. 1E). These results indicate that Akt is expressed in epithelial cells during lactation and is catalytically activated during lactation, suggesting that the decrease of Akt in these cells at the onset of involution may be necessary for apoptosis to proceed.

Myr-Akt Is Expressed in the Mammary Glands of Transgenic Mice

Because endogenous Akt expression decreases during early involution (Fig. 1, A and C), we hypothesized that
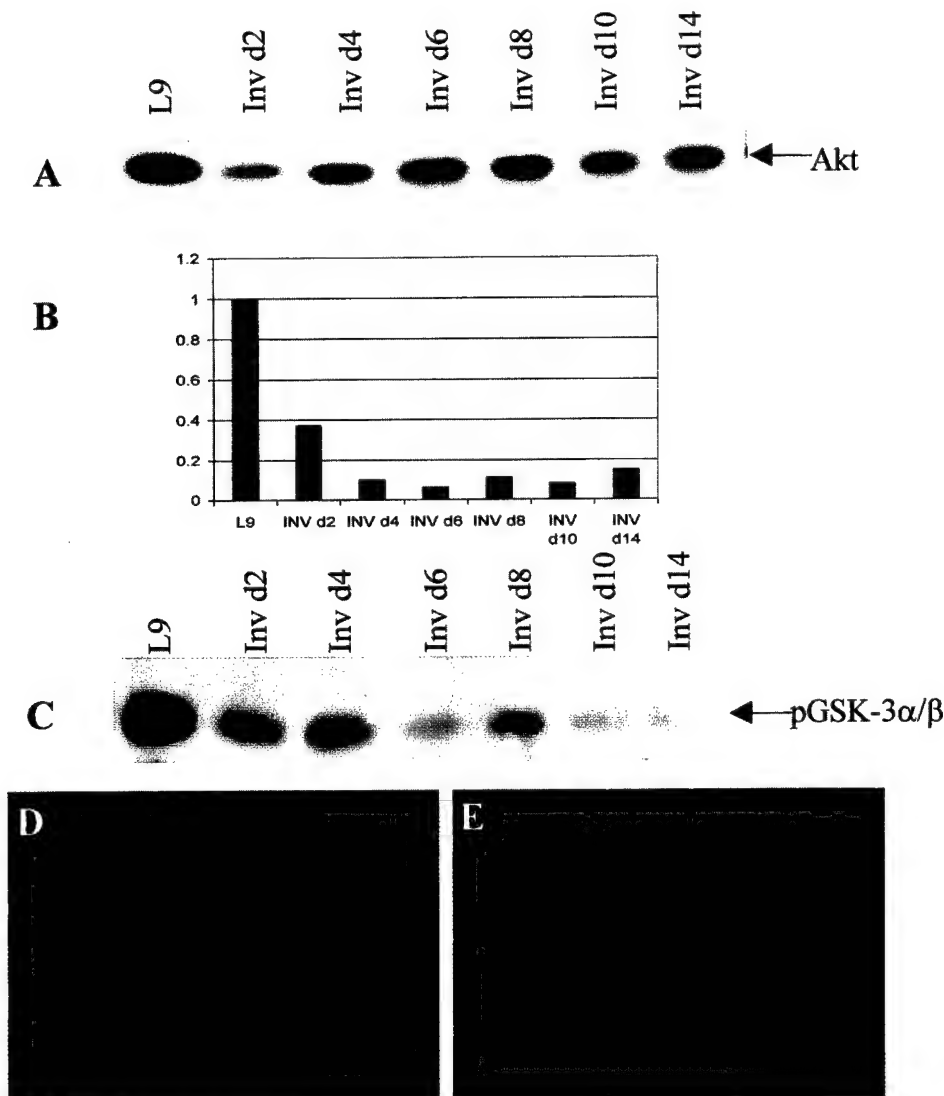
Fig. 1. Akt Expression Decreases during Lactation and Involution

A, Protein extracts were prepared from frozen mammary glands of normal (FVB) mice at day 9 of lactation (L9), and days 2, 4, 6, 8, 10, and 14 of involution (Inv d2, Inv d4, Inv d6, Inv d8, Inv d10, and Inv d14, respectively). The protein extracts were analyzed on an 8% SDS-polyacrylamide gel and immunoblotted with an antibody that recognizes total Akt. B, The Akt and actin immunoblots were scanned on a densitometer and Akt expression was normalized to actin expression. C, Protein extracts were prepared from mammary glands at the indicated times, and the ability of Akt to phosphorylate a GST-GSK3 substrate was examined using a kinase assay. The reactions were analyzed on a 12% polyacrylamide gel and immunoblotted with an antibody that recognizes both phosphorylated isoforms of GSK-3, GSK-3α and -β. D, Sections from a lactating day 9 mammary gland were stained using an anti-Akt1-specific antibody (*red*). *Green* represents the luminal spaces stained with WGA. *Blue* represents the DAPI stained nuclei. E, Negative control of panel D; immunostaining was performed using secondary antibody alone. Panels D and E were taken at a magnification of 200× and exposed for the same length of time.

the inappropriate presence of activated Akt at this stage would result in a change in the involution process. Therefore, we expressed a constitutively active mutant of Akt in the mammary glands of mice under the control of the MMTV promoter, which allows expression of the transgene in the epithelial cells of the mammary gland. Although the MMTV promoter allows expression during all stages of development, the promoter is strongly expressed during lactation. Therefore, we can examine the effect of Akt on the transition between lactation and involution in these transgenic mice. The constitutively active mutant of Akt (myr-Akt) used in these studies contains an amino-terminal myristoylation sequence from c-src fused to the coding sequence of Akt1. This targets the fusion protein to the plasma membrane where it can be constitutively activated after phosphorylation of Thr308 and Ser473 (25). The construct also contains a hemagglutinin (HA) epitope tag at the carboxyl-terminal end of the protein to allow for detection of transgene expression by immunoblotting.

Transgenic mice were produced that express the myr-Akt construct in the mammary gland. Transgene-

positive founder mice were identified by PCR of tail DNA (Fig. 2A). Immunoblot analysis of mammary gland protein for the HA epitope tag indicates that two lines of mice (designated 1173 and 1176) express detectable levels of the myr-Akt protein (Fig. 2B). Expression of the transgene is consistently higher in mice derived from the 1173 founder than expression in mice from the 1176 founder (Fig. 2B and data not shown). These data show that two independently generated lines of MMTV-myr-Akt transgenic mice, lines 1173 and 1176, have been produced that express detectable levels of myr-Akt protein in the mammary gland.

The pattern of expression of the myr-Akt transgene was examined in the transgenic mice during involution. The fourth inguinal mammary glands were collected during lactation and involution from both normal and transgenic mice. Protein extracts were prepared, and immunoblot analysis was performed to detect the HA epitope tag. As shown in Fig. 2C, transgene expression is high during lactation, and expression of myr-Akt persists through day 14 of involution, although transgene levels appear to decrease as involution proceeds. Two bands are apparent on the immunoblot, and this has been consistently observed in all experiments using the anti-HA antibody. Although both bands cross-react with anti-Akt antibodies (data not shown), only the lower mol wt band cross-reacts with the anti-phospho-Akt antibody (Fig. 2D). Phosphorylation of Akt is required for its activation; therefore, levels of phosphorylated Akt in the glands were determined by immunoblotting with an antibody specific to the phosphorylated Ser473 residue of Akt. Two bands were observed in mammary glands from transgene-positive mice (Fig. 2D). The lower mol wt protein corresponds to endogenous Akt and the higher mol wt protein corresponds to the myr-Akt transgene. Phosphorylation of the myr-Akt transgene is high during lactation and early involution and persists through day 14 of involution, although there is a decrease in the amount of phosphorylated transgene throughout involution corresponding to the decrease in total transgene expression. It appears that phosphorylation of endogenous Akt is suppressed by the presence of the myr-Akt transgene from day 9 of lactation (L9) to day 4 of involution (Inv d4) (Fig. 2D, lanes 2–4). To further determine whether the myr-Akt transgene was catalytically active in the mammary gland, the phosphorylation of a known target of Akt, glycogen synthase kinase-3 (GSK-3) (42, 43), was examined. As shown in Fig. 2E, phosphorylation of endogenous GSK-3 is higher in mammary glands from transgenic mice compared with those from normal mice during late pregnancy, lactation, and early involution when transgene expression is high (Fig. 2C and data not shown). Reprobing the immunoblot with an anti-GSK-3α antibody, which also recognizes GSK-3β, indicates that protein levels of GSK-3α/β are not significantly altered in mammary glands from transgenic mice (Fig. 2F). The detection of myr-Akt, its phosphorylation, and the increased phosphorylation

of GSK-3 in the mammary gland indicate that these transgenic mice can be used to examine the effect of activated Akt upon involution.

To localize expression of the transgenic myr-Akt in the mammary gland, immunofluorescence was performed on mammary tissue at day 2 of involution from normal (Fig. 2G) and transgenic (Fig. 2H) mice using an anti-Akt1 antibody. Data presented in Fig. 1B indicate that the level of endogenous Akt is low at this time point. *Red* staining represents Akt1 protein and *blue* staining represents the DAPI-stained nuclei. Some staining is observed in the epithelium of the normal mammary gland (Fig. 2G), which is to be expected because a small amount of Akt protein is observed in normal mammary glands at this stage (Fig. 1B). However, expression of Akt1 in the mammary gland from the transgenic mouse is much greater and is observed in cells surrounding the luminal spaces (Fig. 2H). Identical exposure times were used for photographs in panels G and H of Fig. 2. Sections stained with secondary antibody alone show no staining (data not shown). Therefore, it appears that the myr-Akt transgene is expressed in mammary epithelial cells surrounding the alveolar lumina.

Involution Is Delayed in MMTV-myr-Akt Transgenic Mice

Mammary involution can be divided into an early phase (days 1–4) and a late phase (days 5–8) (8). The early phase of involution is characterized by apoptosis of the alveolar epithelial cells (2). Because Akt can suppress apoptosis, we determined the effect of the myr-Akt transgene upon involution. Involution was induced in both FVB and MMTV-myr-Akt female mice using forced involution as described above. The fourth inguinal mammary glands were removed at days 2, 4, 6, 8, 10, and 14 of involution, fixed, and embedded in paraffin.

Histological features of control and myr-Akt-expressing mammary glands were examined in hematoxylin and eosin-stained sections (Fig. 3). At day 2 of involution, alveolar structures, comprised of a single layer of epithelial cells surrounding a lumen, are observed in mammary glands from both normal and transgenic mice (Fig. 3, A and B). No distinct morphological differences are apparent between the two glands at this stage. At day 4 of involution, the alveolar structures in mammary glands from normal mice have started to collapse, and numerous apoptotic bodies are apparent in the ductal lumens (Fig. 3C). In addition, adipocytes have begun to reappear or, alternatively, to reaccumulate lipid. The predominance of adipocytes is typical of mammary glands in virgin mice and post-involution mice. In the mammary glands from transgenic mice, however, the alveoli have not yet begun to collapse, and they appear distended (Fig. 3D). In addition, there is an accumulation of protein in the alveolar lumina, which may represent inspissated milk typically observed during milk stasis. At day 6 of
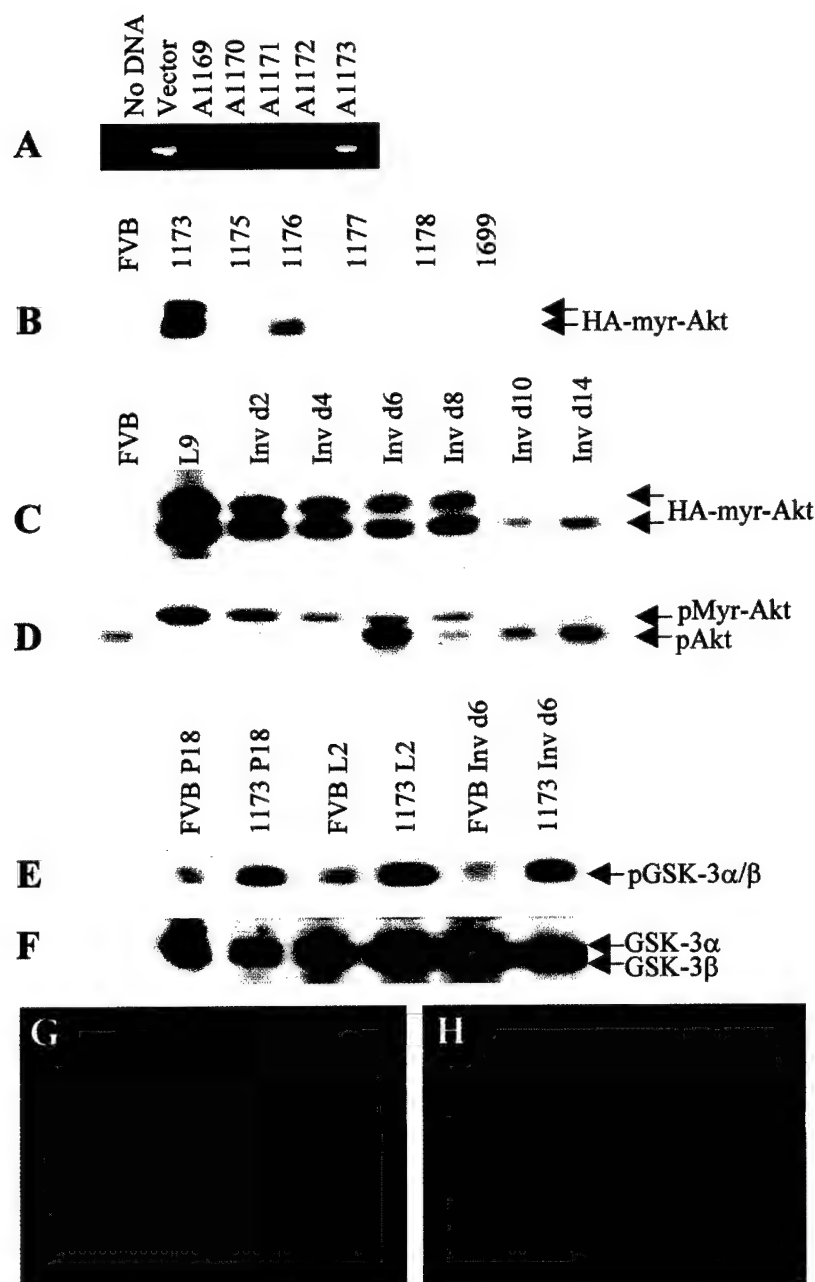
Fig. 2. Generation of MMTV-myr-Akt Mice and Expression of the myr-Akt Transgene

A, Tail DNA was extracted from each founder and PCR analysis was performed using primers specific for Akt and the HA epitope tag. Of 39 founders, 9 contained transgene DNA. B, Protein was extracted from mammary glands at day 18 of pregnancy of normal (FVB) and transgenic (1173, 1175, 1176, 1177, 1178, and 1699) mice and analyzed by immunoblot analysis using an anti-HA antibody. Two lines, 1173 and 1176, show expression of the myr-Akt protein in their mammary glands. C, Protein was extracted from mammary glands at L9, Inv d2, Inv d4, Inv d6, Inv d8, Inv d10, and Inv d14 from both normal (FVB) and transgenic (line 1173) mice. The extracted protein was analyzed by immunoblot analysis using an anti-HA antibody. D, Extracts were immunoblotted with an antiphospho-Akt antibody. The lower mol wt protein corresponds to endogenous Akt and the higher mol wt protein corresponds to the myr-Akt transgene. E, Protein extracted from mammary glands at day 18 of pregnancy (P18), day 9 of lactation (L9), and day 6 of involution (Inv d6) were analyzed by immunoblot analysis using the antiphospho-GSK-3α/β antibody. F, The immunoblot was reprobed with an antibody to GSK-3α. G, A section from a normal gland at day 2 of involution immunostained with an anti-Akt1 specific antibody (*red*). The DAPI-stained nuclei are *blue*. H, A section from a transgenic gland at day 2 of involution immunostained identically to panel G. Panels G and H were taken at a magnification of 200× and exposed for the same length of time.
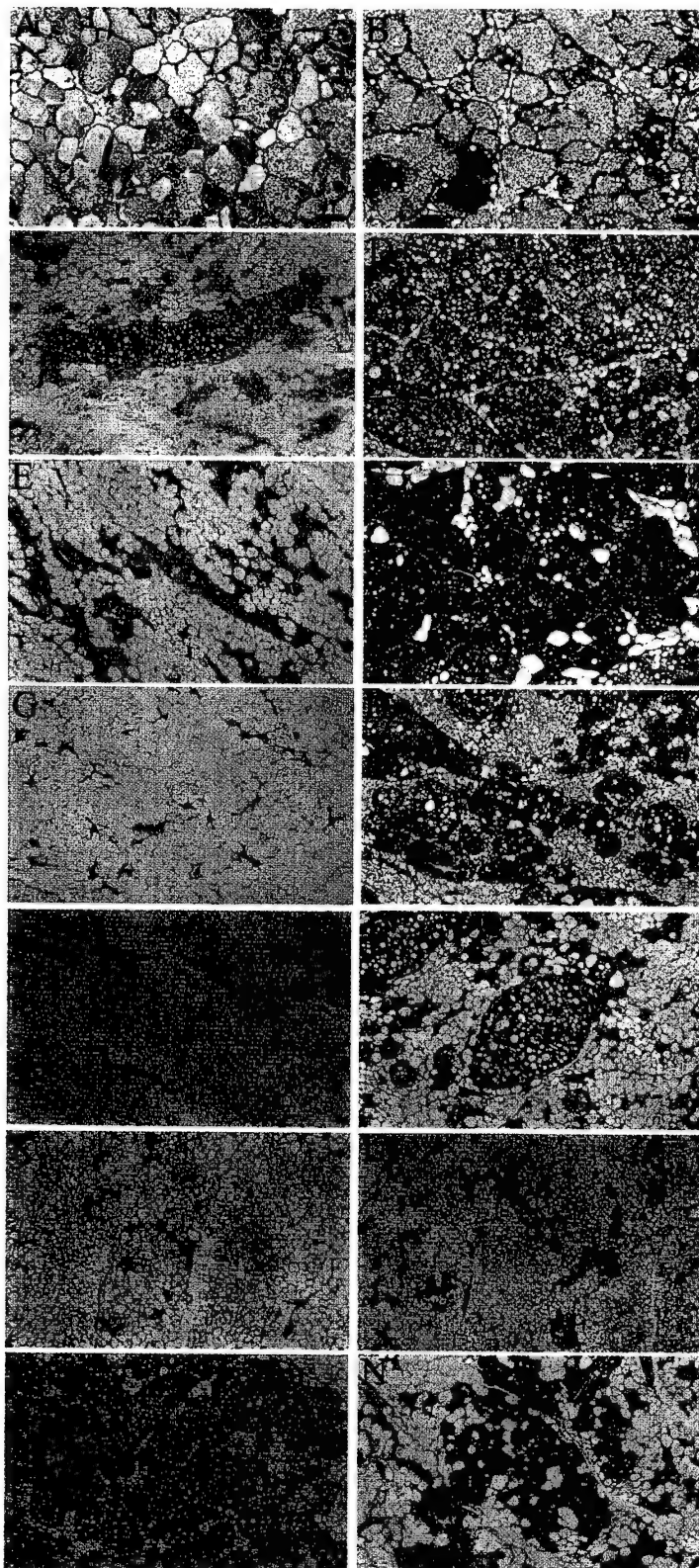
Fig. 3. Involution Is Delayed in Mammary Glands of MMTV-myr-Akt Mice

A, C, E, G, I, and K, Mammary glands from normal (FVB) mice. B, D, F, H, J, and L, Mammary glands from MMTV-myr-Akt mice, line 1173. M and N, Mammary glands from MMTV-myr-Akt mice, line 1176. A and B, Day 2 of involution. C and D, Day 4 of involution. E and F, Day 6 of involution. G and H, Day 8 of involution. I and J, Day 10 of involution. K and L, Day 14 of involution. M, Day 4 of involution. N, Day 6 of involution. *Magnification bars* represent 100 μM.

involution, the alveoli in mammary glands from normal mice have collapsed (Fig. 3E) and the process of re-modeling has continued (8). In mammary glands from transgenic mice, the alveoli have still not collapsed, although some apoptotic bodies are observed in the luminal spaces and some adipocytes have begun to reappear (Fig. 3F), suggesting that the process of in-volution has begun. By day 8 of involution, the normal mammary glands have been completely remodeled and resemble that of a prepregnant gland (Fig. 3G). In the mammary glands from transgenic mice, the alveoli are beginning to collapse and numerous apoptotic bodies are visible in the luminal spaces (Fig. 3H), sim-ilar to the morphology observed at day 4 of involution in the normal gland (Fig. 3C). Normal mammary glands at days 10 and 14 of involution are similar to that seen at day 8, as the mammary gland has been completely remodeled (Fig. 3, I and K). In contrast, mammary glands from transgenic mice are still undergoing the process of involution through day 10 as indicated by the collapsing alveolar structures and the disorganiza-tion of the gland (Fig. 3J). By day 14, the mammary glands from transgenic mice appear to have been remodeled, although some alveolar structures still per-sist (Fig. 3L).

The data shown in Fig. 3 (panels B, D, F, H, J, and L) were from mice derived from the 1173 founder. To determine that the observed phenotype is not due to an insertional artifact of the transgene, these exper-iments were confirmed in mice derived from a sec-ond founder line, 1176. The mammary glands exhibit a similar phenotype as that seen in line 1173, with alveolar structures persisting through day 4 of invo-lution (Fig. 3M). However, alveolar collapse in mam-mary glands from this line begins by day 6 (Fig. 3N) rather than day 8 as seen in the 1173 line (Fig. 3H). This difference in the time course of involution is likely due to the lower expression consistently seen in the mammary glands from the 1176 line compared with those from the 1173 line (Fig. 2B). These data demonstrate that the presence of a constitutively active Akt in the mammary gland delays involution after forced weaning.

Myr-Akt Delays the Onset of Apoptosis during Involution

To determine whether the delayed involution ob-served in the MMTV-myr-Akt mice was accompa-nied by a decrease in apoptosis, the numbers of apoptotic cells were quantified. Figure 4A shows the results of quantifying apoptotic bodies in hematox-ylin and eosin-stained sections of mammary glands from normal and myr-Akt transgenic mice. Consis-tent with observations published previously (7), ap-optosis peaked at day 4 of involution in the mam-mary glands from normal mice. However, the peak of apoptosis in the mammary glands from trans-genic mice did not occur until day 8 of involution,

indicating a delay in the onset of apoptosis in mam-mary glands from transgenic mice.

To confirm these results, TUNEL analysis was per-formed on sections of mammary glands from both normal and transgenic mice. As shown in Fig. 4B, apoptosis in mammary glands from normal mice peaked at days 2–4 of involution. Consistent with Fig. 4A, the peak of apoptosis in mammary glands from transgenic mice did not occur until day 8 of involution. These data indicate that the presence of the myr-Akt transgene results in a delay in the onset of apoptosis during involution.

To compare the epithelial content of the regressed mammary glands from normal and transgenic mice, the amount of epithelium present in mammary glands at day 21 of involution was quantified. As shown in Fig. 4C, epithelial content in mammary glands from trans-genic mice was nearly 2-fold higher than that in mam-mary glands from normal mice. Figure 4D shows rep-resentative pictures of hematoxylin and eosin-stained sections from normal (*upper panel*) and transgenic (*lower panel*) mammary glands at day 21 of involution. These results suggest that less epithelial cells undergo apoptosis in the mammary glands from transgenic mice and that these cells remain until the next pregnancy.

β-Casein and Whey Acidic Protein (WAP) Expression Are Sustained in MMTV-myr-Akt Mice throughout Involution

One of the initial events after weaning is the rapid decrease in the expression of milk proteins such as β-casein and WAP (1). These proteins are highly expressed during lactation, but diminish during the first 5 days of involution (4). We hypothesized that the observed delay in involution would be accom-panied by sustained production of β-casein and WAP RNA. RNA was extracted from the fourth in-guinal mammary glands during lactation and invo-lution, and the levels of β-casein, WAP, and glycer-aldehyde-3-phosphate dehydrogenase (GAPDH) RNA were examined by Northern blot analysis. Ex-pression of β-casein and WAP are sustained in the mammary glands from transgenic mice compared with mammary glands from normal mice (Fig. 5, A and B). β-Casein expression decreases dramatically in the normal gland after weaning, and levels are undetectable by day 6 of involution (Fig. 5A). WAP expression similarly decreases after weaning, and levels are also undetectable by day 6 (Fig. 5B). In contrast, β-casein expression in the mammary glands from transgenic mice remains high and levels are detectable until day 10 of involution, although they are beginning to decrease by day 6 of involution (Fig. 5A). WAP expression is also sustained in the mammary glands from transgenic mice, but de-creases by day 8 of involution (Fig. 5B). It appears that there is more WAP mRNA production at day 2 of involution than during lactation (Fig. 5B); however,
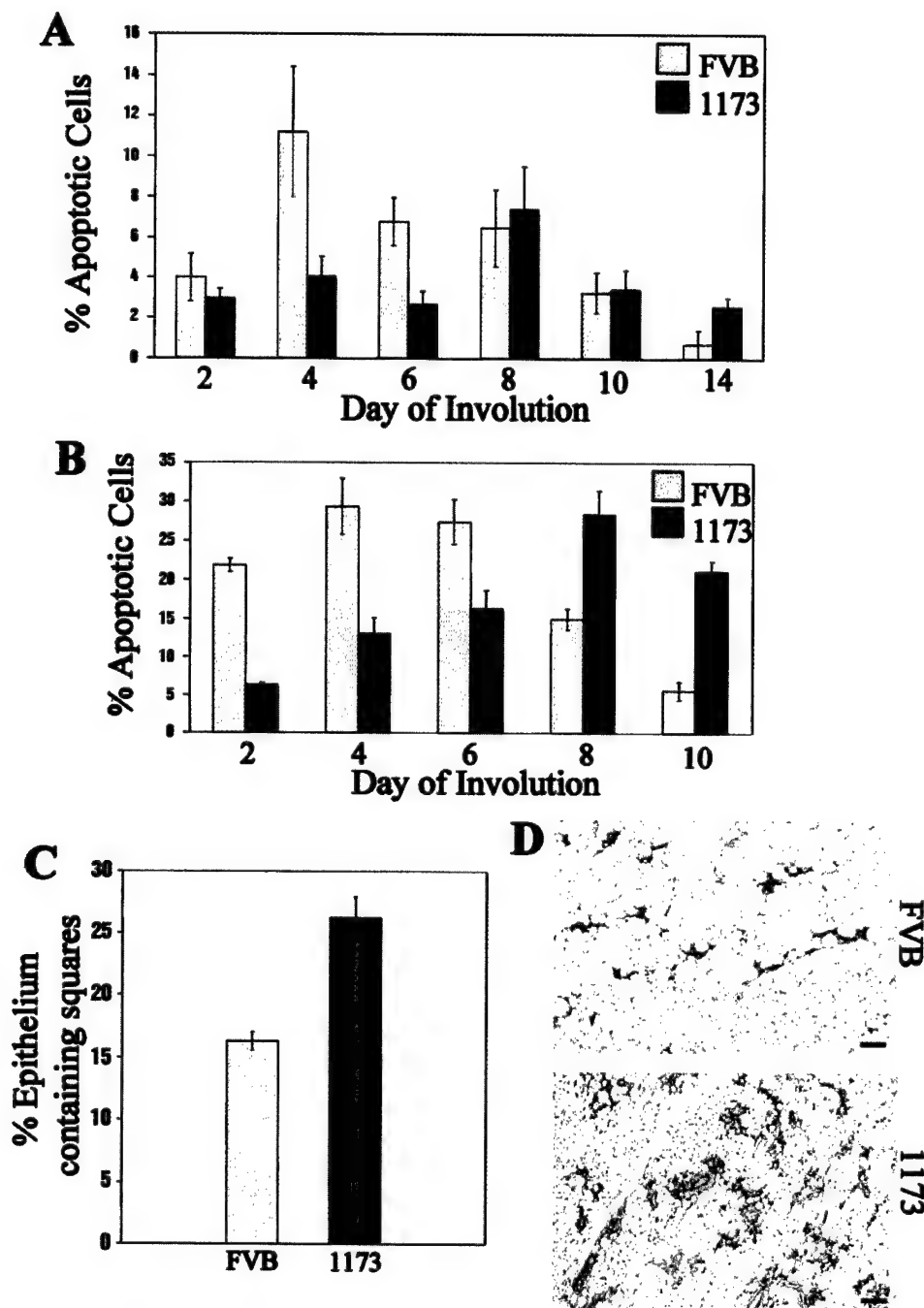
Fig. 4. Delayed Onset of Epithelial Cell Apoptosis during Involution in Mammary Glands of MMTV-myr-Akt Mice

A, Percentage of apoptotic cells in normal mammary glands (*gray bars*) and transgenic mammary glands (*black bars*) at days 2, 4, 6, 8, 10, and 14 of involution assessed in hematoxylin and eosin-stained sections. B, Percentage of apoptotic cells in normal mammary glands (*gray bars*) and transgenic mammary glands (*black bars*) at days 2, 4, 6, 8, and 10 of involution assessed using TUNEL-stained sections. *Error bars* represent SEM. C, Quantification of the epithelial content of mammary glands from 10 normal mice (*gray bar*) and 10 transgenic mice (*black bar*) at day 21 of involution. *Error bars* represent SEM. D, Hematoxylin and eosin-stained sections of mammary glands at day 21 of involution. The *upper panel* is a normal mammary gland from an FVB mouse and the *lower panel* is a section from a mammary gland from line 1173. *Magnification bars* represent 100 μM.

there is a corresponding increase in GAPDH (Fig. 5C), indicating that this is due to a loading artifact. Similar data were obtained in three different studies using RNA isolated from mammary glands of differ-

ent mice. These data demonstrate that milk protein gene expression is sustained in the mammary glands of transgenic mice compared with normal mice.
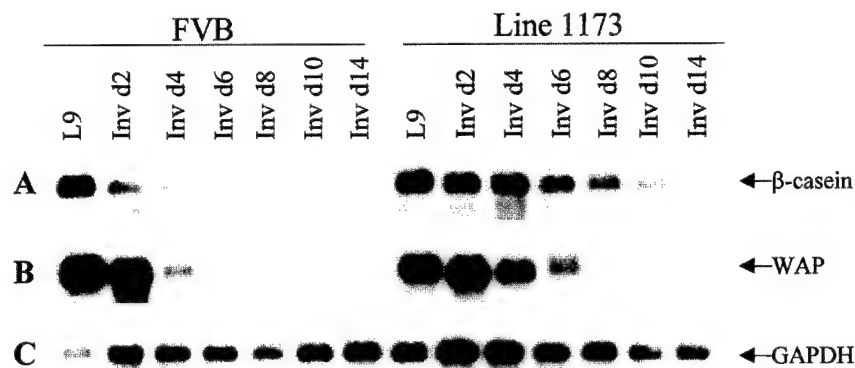
Fig. 5. Milk Protein Expression in MMTV-myr-Akt Mice

Northern blot analysis of total RNA from mammary glands of normal (FVB) and transgenic (line 1173) mice at day 9 of lactation (L9) and days 2, 4, 6, 8, 10, and 14 of involution (Inv). The blots were probed with β-casein (A) and WAP (B) specific probes. C, Blots were reprobed with a GAPDH-specific probe as a loading control.
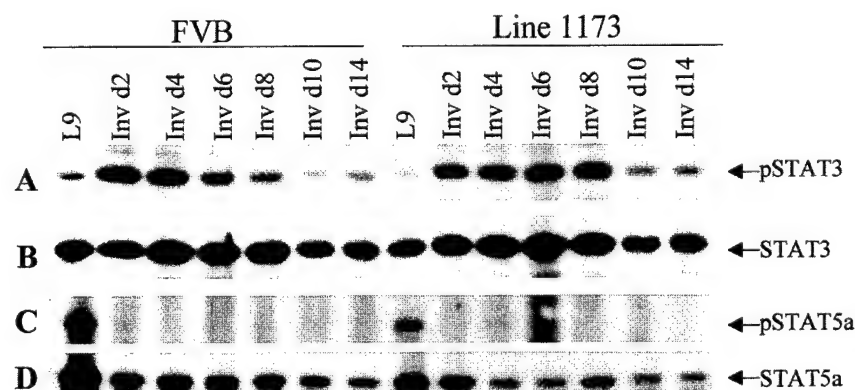

Fig. 6. STAT Activation in MMTV-myr-Akt Mice

Analysis of STAT3 and STAT5a activation in normal (FVB) and transgenic (line 1173) mice at day 9 of lactation (L9) and days 2, 4, 6, 8, 10, and 14 of involution (Inv). A, Protein extracts were analyzed on an 8% SDS-polyacrylamide gel and immunoblotted with a phospho-specific STAT3 antibody. B, The blot was reprobed with an antibody that recognizes total STAT3. C, Protein extracts were analyzed on an 8% SDS-polyacrylamide gel and immunoblotted with a phospho-specific STAT5 antibody. The *arrow* indicates phospho-STAT5a. D, The blot was reprobed with an antibody that recognizes total STAT5. The *arrow* indicates STAT5a.

STAT (Signal Transducer and Activator of Transcription) Expression and Phosphorylation Occur Normally in MMTV-myr-Akt Glands

STATs are a family of transcription factors that are activated by cytokine receptors including the PRL receptor (45, 46). STAT activation requires phosphorylation of a single tyrosine residue, which leads to the formation of homo- and heterodimers that translocate to the nucleus where they regulate the transcription of a number of genes (47). Members of the STAT family have been shown to be involved in normal mammary gland development and function. Specifically, STAT5a is involved in milk production during lactation, and its phosphorylation decreases at the beginning of involution (48, 49). STAT3 is required for normal involution, and its phosphorylation increases at the beginning of involution (9, 49). It has been suggested that this reciprocal regulation may be important for involution to

occur (48, 49). The delayed involution in myr-Akt mice suggests a possible disruption in the normal regulation of these two STAT molecules.

To examine STAT phosphorylation, protein was extracted from mammary glands of both normal and MMTV-myr-Akt mice during lactation and involution. The phosphorylated forms of STAT3 and STAT5a were detected by immunoblot analysis using phospho-specific antibodies. STAT3 phosphorylation is low during lactation, but increases by day 2 of involution in mammary glands from both normal and transgenic mice (Fig. 6A). This increase is transient and decreases by day 8 of involution in mammary glands from normal mice. Phosphorylated STAT3 persists to day 8 in mammary glands from transgenic mice, possibly reflecting the increased numbers of epithelial cells remaining in the mammary glands from transgenic mice. Overall levels of STAT3 are equivalent in mammary glands from both normal and transgenic mice (Fig. 6B).

STAT5a phosphorylation is high during lactation, but decreases by day 2 of involution in mammary glands from both normal and transgenic mice (Fig. 6C). Although there appears to be less phosphorylated STAT5a in the mammary glands from transgenic mice at day 9 of lactation compared with normal glands, detection of total STAT5a antibody reveals less STAT5a protein in the mammary glands from transgenic mice at lactation day 2, possibly due to a loading artifact (Fig. 6D). In addition, there appears to be less STAT5a protein in mammary glands from transgenic mice at days 4 and 6 of involution. However, reprobing this blot with an antiactin antibody reveals that less actin protein is also present (data not shown). This artifact is likely due to the accumulation of milk proteins due to milk stasis that occurs in the transgenic mice through day 6 of involution (Fig. 3, D and F) rather than a specific effect of Akt overexpression on STAT5a protein expression. These data demonstrate that although involution is delayed in the mammary glands of the MMTV-myr-Akt mice, the patterns of STAT3 activation and STAT5a inactivation do not change, although STAT3 phosphorylation does persist longer in the transgenic mice. Similar data were obtained in three different studies using extracts prepared from different mice.

Increased and Prolonged TIMP-1 Expression in MMTV-myr-Akt Glands

After apoptosis of the secretory epithelial cells during involution, the mammary gland is remodeled back to a prepregnant state. This process involves activation of MMPs, a family of enzymes that degrades extracellular matrix, and the inactivation or decrease in the concentration of their inhibitors, the TIMPs (4, 6). Expression patterns of the MMP stromelysin-1 (MMP-3) and its inhibitor TIMP-1 during mammary gland involution

have been well characterized (4, 50). TIMP-1 is expressed at moderate levels during lactation, but increases in early involution with a peak of expression at 4 days of involution (50). MMP-3 is undetectable during lactation, but increases during involution with a peak of expression at 4–6 days of involution (50). The ratio of MMP to TIMP expression is important for the remodeling process to occur, and disruption of this ratio has been shown to alter involution. Implantation of TIMP-1 pellets in the mammary gland results in delayed involution (4), and overexpression of MMP-3 in transgenic mice results in precocious involution (51, 52).

To determine whether expression of MMP-3 and TIMP-1 are altered in the MMTV-myr-Akt mice, the amount of TIMP-1 and MMP-3 RNAs was examined using Northern blot analysis. TIMP-1 expression in mammary glands from normal mice increases during early involution, peaking at days 2–4 and decreasing by day 8 of involution (Fig. 7A). In mammary glands from transgenic mice, TIMP-1 levels also increase during early involution but remain high until day 10 of involution (Fig. 7A). GAPDH levels are equivalent in mammary glands from both normal and transgenic mice (Fig. 7B). MMP-3 expression in mammary glands from normal mice is low during lactation and increases by day 4 of involution, peaking at days 4–6, followed by a subsequent decrease in expression by day 10 of involution (Fig. 7C). In transgenic mice, MMP-3 levels increase earlier than in normal mice and are detectable on day 2 of involution, peaking at day 6, and are followed by a decrease in expression at day 10 of involution. The observed earlier increase of MMP-3 in mammary glands from transgenic mice is not consistent with a delay in involution. However, since the MMP to TIMP ratio is thought to be critical for MMP activity, the MMP-3 activity is likely being suppressed
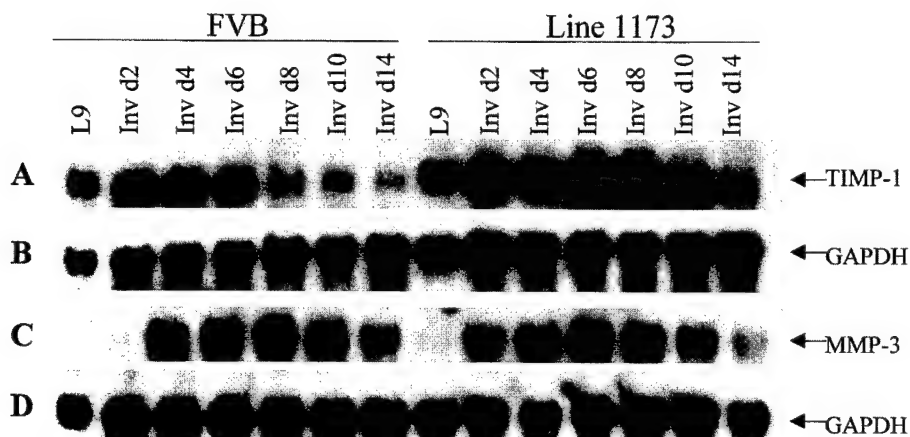

Fig. 7. TIMP-1 and MMP-3 Expression in MMTV-myr-Akt Mice
Northern blot analysis of total RNA from mammary glands of normal (FVB) and transgenic (line 1173) mice at day 9 of lactation (L9) and days 2, 4, 6, 8, 10, and 14 of involution (Inv). Blots were probed with a TIMP-1 specific probe (A) and reprobed with a GAPDH specific probe as a loading control (B), a MMP-3-specific probe (C), and re-probed with a GAPDH specific probe as a loading control (D).

by the high levels of TIMP-1 at this stage (Fig. 7A). GAPDH levels are equivalent in mammary glands from both normal and transgenic mice (Fig. 7D). Similar results were obtained in three different studies using tissue extracts prepared from different mice. These data suggest that the increased and sustained levels of TIMP-1 in the mammary glands of transgenic mice may be involved in the delayed involution observed in the myr-Akt transgenic mammary glands.

DISCUSSION

The data presented here suggest a role for Akt in normal mammary gland involution and demonstrate suppression of apoptosis by Akt in an *in vivo* system. Expression of endogenous Akt was examined during involution induced by forced weaning and, after normalization to actin, was found to be high during lactation and decreased at all days following pup withdrawal. Akt1 expression was observed in lactating glands to be primarily located in the luminal epithelial cells. It would be of great interest to determine whether other cell types in the mammary gland express Akt, as well as whether different cell types express different isoforms.

We have generated transgenic mice that express a constitutively active Akt in the mammary gland using the MMTV promoter. These mice were shown to express the transgene during lactation as well as during the first 8 days of involution, with detectable levels remaining at day 14 of involution. Morphologically, the mammary glands of the transgenic mice show a delay in involution that corresponds with a delay in the onset of apoptosis. The mammary epithelial cells eventually undergo apoptosis, and the gland is remodeled suggesting that either transgene expression diminishes and can no longer suppress apoptosis, or that another apoptotic mechanism is present that can override the effects of the myr-Akt transgene.

Understanding the suppression of apoptosis is of great relevance to cancer, as overexpression of genes that suppress apoptosis lead to cancers such as leukemia (53). Although the mammary glands of the MMTV-myr-Akt mice eventually undergo involution, it appears that less epithelial cells are lost than in normal regressed mammary glands. This observation raises the possibility that the presence of the myr-Akt transgene in mammary glands may result in the retention of epithelial structures, particularly in uni- and multiparous mice, which could lead to the development of hyperplasia and/or tumors.

One of the initial characteristics of involution is the decrease in the production of milk proteins in early involution (1). β-Casein and WAP mRNA expression were sustained in the mammary glands of the transgenic mice compared with those of normal mice. In the histological sections (Fig. 3), it is apparent that the alveolar structures are retained in the glands from

transgenic mice through day 6 of involution whereas the alveolar structures in glands from normal mice regress after day 2 of involution. It has been demonstrated that association of mammary epithelial cells with extracellular matrix is important for maintaining the differentiation status of the epithelial cells and that disruption of this interaction results in the loss of β-casein expression (4). Therefore, it is possible that the epithelial cells that have not undergone apoptosis in the mammary glands from transgenic mice are still associated with the extracellular matrix, resulting in the maintenance of their differentiation state as suggested by the prolonged expression of milk protein genes.

STATs have been shown to be important regulators of mammary gland development. STAT5a knockout mice have impaired lobuloalveolar development during pregnancy and fail to lactate (54). STAT5a is activated during pregnancy and is involved in milk protein expression (48, 49, 55), but its activity decreases at the beginning of involution and has been suggested to be involved in the suppression of apoptosis of epithelial cells (9). As mentioned in the *Introduction*, STAT3 is required for normal involution as demonstrated by delayed involution in conditional knockouts (9). STAT3 activity is low during lactation and increases at the beginning of involution (9, 49). It has been suggested that STAT5 and STAT3 are reciprocally regulated and that this regulation may be important for the normal process of involution (9, 49). Because of the observed delay of involution in the myr-Akt mice, we examined the phosphorylation states of both STAT3 and STAT5a. Although phosphorylated STAT3 persists longer in mammary glands from transgenic mice, this may be due to higher epithelial cell content and the observed delay in involution. Both molecules were expressed equally at the protein level in mammary glands from both normal and transgenic mice. These data may suggest that either Akt functions independently of STAT3 to suppress apoptosis, or that Akt functions downstream of STAT3.

Studies have shown that STAT5a can induce the transcription of the milk proteins such as β-casein (48, 56). Although β-casein levels remain high after weaning in the myr-Akt mice, phosphorylated STAT5a levels decrease by day 2 of involution. Therefore, the increased level of β-casein RNA observed in mammary glands from transgenic mice could reflect either continued transcription of the gene in the absence of phosphorylated STAT5a or an increase in the half-life of the mRNA.

MMPs and their inhibitors, the TIMPs, are critical for the process of remodeling of the gland after apoptosis (4, 6). TIMP-1 has been demonstrated to increase at the beginning of involution, presumably to inhibit the remodeling process until after apoptosis has occurred, and then decrease to allow the MMPs to begin the remodeling process (50). TIMP-1 expression increases during involution in the MMTV-myr-Akt mice, and it remains elevated until day 10 of involution. Interest-

ingly, the expression pattern of MMP-3 in the MMTV-myr-Akt mammary glands is similar to that of the normal glands, although its expression is increased earlier in the mammary glands from transgenic mice when compared with control mice. It is possible that in response to the inability of the epithelial cells to undergo apoptosis, the mammary glands from transgenic mice are up-regulating pathways involved in inducing apoptosis and remodeling in an attempt to overcome this phenotype. It would be interesting to determine the expression patterns of other proteins known to be involved in inducing apoptosis in the mammary gland. Although MMP-3 is expressed earlier in the mammary glands of transgenic mice induced to undergo involution, the increased and sustained levels of TIMP-1 may inhibit MMP-3 activation. It would be interesting to determine whether the expression of other MMPs is altered in our transgenic mice and whether they are responsible for the remodeling that eventually occurs in the mammary glands of myr-Akt transgenic mice.

The coordination of apoptosis and tissue remodeling during involution is not completely understood. One explanation for the delayed involution observed in the mammary glands of transgenic mice is that myr-Akt suppresses apoptosis of the secretory epithelial cells; therefore, the signals required to initiate the remodeling process may not be present. An additional factor contributing to this phenotype may be the observed alteration in MMP to TIMP ratios. The ratio of TIMPs to MMPs is critical for normal involution. Talhouk et al. (4) used pellet implants to demonstrate that the presence of high levels of exogenous TIMP protein during involution resulted in delayed regression of alveolar structures and sustained expression of β-casein, demonstrating that the TIMP to MMP ratio is important for normal involution and regulation of mammary epithelial function. Therefore, the altered ratio of TIMP to MMP might explain not only the delay in tissue remodeling, but also the sustained expression of β-casein in the transgenic mammary glands. One possible mechanism for the increased levels of TIMP-1 is that Akt may induce TIMP-1 transcription or alter the molecules that regulate TIMP-1 gene expression. Another possibility is that activated Akt may be indirectly regulating TIMP-1 expression by modulating a signal from the apoptotic mammary epithelial cells that is normally required to down-regulate TIMP-1.

A recent study by Fata et al. (57) has implicated Akt activity in alveolar development during pregnancy. Impaired alveolar development in osteoprotegerin-ligand (OPGL) knockout mice correlated with loss of phosphorylated Akt. Expression of OPGL by pellet implants in pregnant mammary glands results in an increase in phosphorylated Akt and the restoration of alveolar development. The data presented in this paper focus on the antiapoptotic functions of Akt in the mammary gland and indicate that the loss of Akt at weaning is necessary for normal involution to occur. We have shown that expression of activated Akt in the mammary gland results in suppression of apoptosis and

delayed involution. The delayed involution may be a result of increased TIMP-1 expression, and future studies will focus on the regulation of TIMP-1 expression in these mice. In addition, the identification of Akt targets will define other molecules involved in regulating both apoptosis and tissue remodeling. Clearly, the overexpression of signaling molecules in transgenic mice can reveal potential roles in normal developmental processes, but this analysis may be prone to artifacts. Confirmation that Akt is critical in regulating mammary gland involution will require analysis of knock-out mice that eliminate expression of Akt1, Akt2, or both.

MATERIALS AND METHODS

Generation of Transgenic Mice

The HA-tagged myr-Akt (Akt1) construct was obtained from Dr. Phil Tsichlis (Kimmel Cancer Center, Temple University, Philadelphia, PA) and the MMTV-SV40-BSSK plasmid was obtained from Dr. William Muller (McMaster University, Ontario, Canada). The HA-tagged myr-Akt construct was isolated from the CMV-6 plasmid using *Hind*III and *Eco*RI. The fragment was subcloned into the same sites of the MMTV-SV40-BSSK plasmid. The MMTV-myr-Akt plasmid was digested with *Sal*I/*Spe*I to remove the plasmid backbone and gel purified using an ElutipD column (Schleicher & Schuell, Inc., Keene, NH) according to the manufacturer's instructions. Transgenic mice were generated using standard techniques (58). Mice were maintained on an FVB/N background using stock mice from Taconic Farms, Inc. (Germantown, NY). Tail DNA was extracted from the founder mice by Proteinase K digestion followed by phenol-chloroform extraction and ethanol precipitation. Transgenic mice were identified using PCR. The primers used were 5'-GCCGCTACTATGC-CATGAAGA-3', which is specific to Akt and 5'-GTAATCTG-GAACATCGTATGGGTA-3', which is specific to the HA tag.

Tissue Collection

Adult female mice were mated. Litters were normalized to eight pups and were removed after 9 days of lactation. The females were killed by cervical dislocation at day 9 of lactation, or days 2, 4, 6, 8, 10, and 14 of involution. The tissues were either snap frozen in dry ice for RNA and protein extraction or were fixed in 10% neutral buffered formalin and embedded in paraffin. The embedded tissues were sectioned at 5 μM and stained with hematoxylin and eosin. Mammary glands were removed from at least five mice per time point for histological analysis, and all subsequent analyses were performed in duplicate on tissue from at least three mice per time point.

Assessment of Epithelial Cell Apoptosis

Apoptotic cells were quantified using hematoxylin and eosin-stained sections based on morphological criteria (59). The number of apoptotic cells was calculated as a percentage of total cell counts. TUNEL staining was performed on sections using the Apoptag kit (Intergen, Purchase, NY) following the manufacturer's instructions. The number of apoptotic cells was calculated as a percentage of total cell counts. Hematoxylin and eosin-stained sections were used to calculate epithelial content of glands at day 21 of involution. Epithelial content was quantified by overlaying a grid consisting of 384

squares onto images of mammary glands taken at 100× magnification. The numbers of squares containing epithelium were counted and calculated as a percentage of the total number of squares. The resulting percentage is a representation of the area of the gland containing epithelium rather than a direct count of epithelial cells. A total of 12 mammary glands at day 21 of involution, 6 glands from normal mice and 6 glands from transgenic mice, were quantitated. The study was performed blindly.

Northern Blot Analysis

RNA was extracted from frozen tissue using Trizol (Life Technologies, Gaithersburg, MD). The RNA was then denatured and analyzed on a 1% agarose gel containing 6% formaldehyde and transferred onto GeneScreen (NEN Life Science Products, Boston, MA) according to manufacturer's protocols. Probes for the Northern blots were radiolabeled using random primers (Prime-It II, Stratagene, San Diego, CA) and $[\alpha-^{32}P]$-dCTP (NEN Life Science Products). The β-casein probe was obtained from Dr. Lothar Hennighausen (NIH, Bethesda, MD). The WAP probe was obtained from Dr. Jeffrey Rosen (Baylor College of Medicine, Houston, TX). The TIMP-1 probe was obtained from Dr. Zena Werb (University of California San Francisco, San Francisco, CA). The stromelysin-1 (MMP-3) probe was obtained from Dr. Lynn Matrisian (Vanderbilt College of Medicine, Nashville, TN). The GAPDH probe was from the collection of this laboratory.

Immunoblot Analysis

Protein was extracted from frozen tissue by homogenizing in RIPA lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 1% Triton X-100, 1% DOC, 0.1% SDS, 1 mM dithiothreitol, 5 mM sodium orthovanadate, 100 μg/ml phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail (no. P 8340, Sigma, St. Louis, MO). The samples were then boiled for 10 min, chilled on ice, and sonicated until homogeneous. Protein assays were performed using the Pierce Coomassie Plus protein assay reagent (Pierce Chemical Co., Rockford, IL). Total protein (50 μg amounts) was separated on 8% SDS-polyacrylamide gels, transferred to PVDF membrane (Immobilon, Millipore Corp., Bedford, MA), and immunoblotted with various antibodies as described previously (60, 61). The anti-Akt, antiphospho-Akt, antiphospho GSK-3α/β, antiphospho-STAT3, and anti-STAT3 rabbit polyclonal antibodies were obtained from New England Biolabs, Inc. (Beverly, MA). The antiactin and anti-GSK-3α antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The antiphospho-STAT5 rabbit polyclonal antibody was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). The anti-STAT5 rabbit polyclonal antibody was obtained from Transduction Laboratories, Inc. (Lexington, KY). The horseradish peroxidase-conjugated anti-HA antibody (clone 3F10) was obtained from Roche Molecular Biochemicals (Indianapolis, IN). All antibodies were used at a dilution of 1:1000, except the anti-HA antibody, which was used at a dilution of 1:400. Bound antibodies were detected using horseradish peroxidase-conjugated secondary antibody followed by detection with the ECL system according to manufacturer's recommendations (Amersham Pharmacia Biotech, Piscataway, NJ). Densitometric analysis was performed on the Computing Densitometer, and images were quantitated using ImageQuant software, both from Molecular Dynamics, Inc. (Sunnyvale, CA).

Akt Kinase Assay

Mammary glands were removed from animals at the indicated times and snap frozen in liquid nitrogen. Lysates were prepared by homogenizing the tissue in lysis buffer [150 mM

NaCl, 50 mM HEPES, pH 7.4, 2 mM EGTA, pH 8.0, 1% Triton X-100, 0.25% deoxycholate, and a protease inhibitor cocktail (no. P 8340, Sigma, St. Louis, MO)]. The samples were centrifuged at 13,000 × g for 30 min and the supernatants were used for immunoprecipitation. Protein assays were performed using the Pierce Coomassie Plus protein assay reagent (Pierce Chemical Co.). One milligram amounts of total protein were used for each immunoprecipitation. The immunoprecipitation and kinase assays were performed using the Akt Kinase Assay Kit (no. 9840, New England Biolabs, Inc.) following the manufacturer's instructions. The reactions were analyzed on a 12% polyacrylamide gel and immunoblotted as described above using the antiphospho-GSK-3α/β antibody.

Immunofluorescence

Mammary glands were removed from animals at the indicated times, fixed in 10% neutral buffered formalin, and embedded in paraffin. Sections were cut at 5 μm. After dehydration with graded alcohols, microwave antigen retrieval was performed for 20 min in 10 mM sodium citrate, pH 6.0. The sections were permeabilized with 0.2% glycine in PBS, blocked with 5% normal goat serum, and incubated with the anti-Akt1 antibody kindly provided by Dr. Morrie Birnbaum (University of Pennsylvania, Philadelphia, PA) at a dilution of 1:200. The sections were then incubated with Cy3-conjugated antirabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), Oregon-Green 488-conjugated WGA (Molecular Probes, Inc., Eugene, OR), and 0.6 μg/ml DAPI (Sigma). Images were collected using SlideBook software (Intelligent Imaging Innovations, Inc., Denver, CO) on a Diaphot TMD microscope (Nikon, Melville, NY) equipped for fluorescence with a Xenon lamp and filter wheels (Sutter Instruments, Novato, CA), fluorescent filters (Chroma, Brattleboro, VT), cooled CCD camera (Cooke, Tonawanda, NY), and stepper motor (Intelligent Imaging Innovations, Inc., Denver, CO).

Acknowledgments

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